

A Thesis Submitted for the Degree of PhD at the University of Warwick

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RETROVIRUSES AND INSECT CELLS

A thesis submitted for the degree
of Doctor of Philosophy
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DECLARATION

I declare that this thesis has been composed entirely by myself and that it has not been used in any previous application for any degree. All work represented herein is my own unless specifically acknowledged. All sources of information have been specifically acknowledged by means of references in the text.

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I would like to thank my supervisor in Warwick, Dr. Roger Avery, for his support and guidance throughout this work and his continuing friendship. My thanks to Dr. David Kelly in Oxford for his help during a very enjoyable stay there and subsequent discussions.

My thanks also go to Prof. Derek Burke for the provision of a virus group with such a friendly atmosphere, the members of the Tumour Virus Laboratory, especially Jenny Jones, for help and advice and to the Science Research Council for the provision of funds.

Grateful thanks to Mrs. Mary Alexander for typing this thesis against all adversity - baby included.

Finally my thanks to my wife, Anne, for her encouragement, advice, help with the English language and for being there.

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"O God of battles, steel my soldiers' hearts,
Possess them not with fear! Take from them now
The sense of reck'ning, if th' opposed numbers
Pluck their hearts from them!"

King Henry the Fifth Act IV Scene I

William Shakespeare

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SUMMARY

Several Invertebrate cell lines were examined for the presence of retrovirus particles. When cells of a Drosophila melanogaster cell line were disrupted and analysed on sucrose density gradients, a subcellular fraction with a density of 1.22 g/ml was found to possess endogenous DNA polymerase activity and could catalyse polymerization of deoxynucleotide triphosphates in response to added template primers. The latter activity had the cation and template primer responses expected for reverse transcriptase. A high molecular weight polyadenylic acid-containing RNA was also purified from this fraction and could be dissociated by heat treatment into 30 to 35S and smaller species. Electron microscopy revealed the presence of toroidal forms reminiscent of intracytoplasmic A-type retrovirus particles within the Drosophila cells. Similar forms were found associated with the subcellular fraction of 1.22 g/ml.

Drosophila cells could be induced by halogenated pyrimidines to release a polymerase activity capable of utilizing the template primer poly(rA).oligo(dT). Pellets were prepared from induced cell culture fluids and analysed on sucrose gradients. Two peaks of poly(rA).oligo(dT) utilization were obtained of density 1.14 and 1.20 g/ml. Radiolabelled RNA was prepared from these fractions. Radiolabelled material from both 1.14 and 1.20 g/ml density fractions sedimented at a position corresponding to 60-70S molecular weight RNA. Those high molecular weight RNAs could be dissociated by heat treatment into 30-35S species.

Infection of insect cells with known Avian and Murine retroviruses was carried out. No positive results could be obtained by using

polymerase activity as a measure of replication. By using Southern blot analysis, it was found that Drosophila cells could acquire exogenous viral sequences from XLV (KMSV).

A number of cell lines were treated with the induced agent from Drosophila cells in an attempt to demonstrate infectivity. This could not be detected by the use of poly(rA).oligo(dT) utilization as a measure of replication.

These results are discussed and conclusions are drawn.

ABBREVIATIONS

ALSV	Avian leukosis/sarcoma viruses
AMV	Avian myeloblastosis virus
ASV	Avian sarcoma virus
BDUR	Bromodeoxyuridine
b. p.	Base pair
CAT	Canine thymus cells
CEF	Chick embryo fibroblast cells
Ci	Curie (3.7×10^{10} disintegrations per second)
cpm	Counts per minute
d	Dalton
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DMEM	Dulbecco's Modification of Eagles Medium
DMSO	Di methyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
FBS	Foetal Bovine Serum
Δ FBS	Heat inactivated Foetal Calf Serum
g	Gravity
HaMSV	Harvey Murine Sarcoma Virus
IDUR	Iododeoxyuridine
k. b.	Kilobase
KMSV	Kirsten Murine Sarcoma Virus
M	Molar
MMTV	Mouse Mammary Tumour Virus
MPMV	Mason-Pfizer Monkey Virus

mRNA	Messenger Ribonucleic acid
MSV	Murine Sarcoma Virus
m. w.	Molecular Weight
N	Normal
NIH	National Institutes of Health
NRK	Normal Rat Kidney
oligo(dG)	Oligodeoxyguanylate
oligo(dT)	Oligodeoxythymidylate
p	Passage
PBS	Phosphate buffered saline
pfu	Plaque forming unit
poly(dA)	Polydeoxyadenylic acid
poly(rA)	Polyadenylic acid
poly(rC)	Polycytidylic acid
poly(rCm)	Polymethylcytidylate
POPOP	1,4 - di(2(5-phenyl-oxazolyl))-benzene
PPO	2,5 - diphenyloxazole
RNA	Ribonucleic acid
RNase	Ribonuclease
RSV	Rous Sarcoma Virus
S	Svedberg unit
SDS	Sodium dodecyl (lauryl) sulphate
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl) aminomethane
TTP	Thymidine 5'-triphosphate
UV	Ultraviolet
v/v	Volume for volume
w/v	Weight for volume
w/w	Weight for weight
XLV	Xenotropic leukemia virus

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INTRODUCTION

Taxonomy

There are four characteristics which define the virus Family Retroviridae. These are:

- a) Architecture of the virion;
- b) Diploid, single-stranded RNA genome;
- c) Presence of reverse transcriptase;
- d) Requirement for DNA intermediate in viral replication.

These characteristics are discussed in detail below.

The Family Retroviridae contains three subfamilies; the Oncovirinae, Spumavirinae and the Lentivirinae (Fenner, 1976). The Spumavirinae or Foamy Viruses are viruses that appear to have no cytopathic or pathological effect on the host cell. The Lentivirinae are viruses which induce chronic degenerative disease with gross cytopathic effects within the host. Examples of this group are Maedi and Visna viruses. The Oncovirinae or RNA tumour viruses may be further classified according to any of the following characteristics:

- a) Morphology: the Oncornaviruses may be of A, B, C or D- type.

These properties will be discussed later.

- b) Endogenous/exogenous: RNA tumour viruses may be endogenous or exogenous to the host. A virus which has its genome contained within the gametes or germ line of the host, and which may be transmitted vertically to the host progeny is termed endogenous. An exogenous virus is one which spreads by horizontal or epigenetic transmission to other susceptible hosts. The species of origin may be defined by DNA hybridization data.

- c) Host Range: the host range of the viruses may be used as a means of classification. The murine leukemia viruses (MLV) may be grouped as ecotropic, xenotropic or amphotropic (Gk: oikos, home; tropos, turning; xenos, foreigner; ampos, both). Ecotropic MLV may be further classified as N or B tropic (see later). Avian viruses may be classified as subgroups of leukosis - sarcoma viruses.
- d) Antigenic composition: various antigens are found in the Oncornaviruses. The group -, type -, and interspecies antigens may be used as a graded form of classification.
- e) Oncogenic properties: RNA tumour viruses may be divided into two major groups on the basis of their oncogenic properties. The first group, classed as highly oncogenic or highly transforming, consists of those viruses with the ability to transform fibroblastic cells in vitro or induce sarcomas in susceptible hosts, eg MSV, ASV. The second group are the weakly oncogenic or weakly transforming viruses eg non-defective ALV and MLV which produce, predominantly, forms of leukemia in susceptible hosts after a considerable latent period. Exceptions to these definitions are the Abelson virus which transforms fibroblasts and induces acute lymphomas in vivo, (Scher & Siegler 1975), and the Mink cell focus forming strains of MLV, (Hartley et al, 1977). It is common for all 5 forms of classification to be used when discussing these viruses.
- In this work the Type -C RNA tumour viruses, the best characterized type of the Retroviridae, were studied.

Historical Background

In 1908, Ellerman and Bang discovered that cell-free filtrates from leukemic chickens, on injection into healthy birds, induced the same disease as in the original host. The infectious agent was later shown to be an RNA tumour virus. Rous reported a transmissible sarcoma of chickens in 1911 which was later shown to be caused by a virus. Mammals were reported to harbour RNA tumour viruses by Bittner (Bittner, 1936) working on the mammary tumour of mice. His findings indicated that tumour incidence was correlated with nursing and it was soon discovered that the agent responsible was passed, from mother to offspring, in the milk. Thus a second animal species had been shown to harbour RNA tumour viruses.

Fifteen years after the publication of Bittner's work, Ludwig Gross discovered a murine leukemia virus (Gross, 1951) whilst working with the AKR inbred strain of mice. He transferred extracts of spontaneous lymphomas from these mice into new-born C3H/B1 mice. Tumours were induced at the site of inoculation followed by leukemia. Lymphoid tumours were passed back into AK mice and, after several passages, the Gross passage A leukemia virus was isolated from these leukemic mice.

Harvey in 1964 and Moloney in 1966 were the first workers to report the discovery of sarcoma-inducing viruses of mice. The method used to isolate such viruses was to pass Moloney leukemia virus through rodents and to use the cell fluid to inoculate new-born Balb/c mice.

By a similar process, that is passing murine erythroblastosis virus through rats, Kirsten and Mayer isolated another strain of sarcoma virus in 1967.

Subsequently, RNA tumour viruses have been identified in a wide variety of animal hosts including fish, snake, mink, cow, gibbon and baboon, see Table 1. As yet, however, they have not been isolated from invertebrates.

TABLE 1

Some animal hosts of RNA tumour viruses

Baboon	Beveniste <u>et al</u> , 1974
Cat	Todaro <u>et al</u> , 1973
Chicken	Rous, 1911
Cow	Rohde <u>et al</u> , 1978
Gibbon	Kawakami <u>et al</u> , 1972
Goose	Bauer & Temin, 1979
Mink	Barbacid <u>et al</u> , 1978
Mouse	Gross, 1958
Pig	Moennig <u>et al</u> , 1974
Pike	Papas <u>et al</u> , 1976
Rat	Rasheed <u>et al</u> , 1978
Rhesus monkey	Chopra & Mason, 1970
Ring necked pheasant	Tereba, 1979
Spectacled langur	Todaro <u>et al</u> , 1978
Squirrel monkey	Heberling <u>et al</u> , 1977
Tree shrew	Flügel <u>et al</u> , 1978
Turkey	Yann & Gazit, 1979
Viper	Clark <u>et al</u> , 1974
Woolly monkey	Theilen <u>et al</u> , 1971

Ultrastructure and Nomenclature

The RNA tumour viruses are spherical, enveloped particles, 90-110nm in diameter. They possess an inner electron-dense nucleoid which can vary in position and electron diffraction properties - dependent upon the virus type, (A, B, C or D). The morphology of the particle and the way in which it matures, determines its type (Bernhard & Guérin, 1958; Bernhard 1960; de Harven 1975; Dalton 1972 (a); Dalton 1972 (b)).

Short spikes and/or knobs are often visualized on the surface of type B and C particles - these being especially clear on the B-type viruses.

Type -A Particles

These RNA tumour viruses should strictly be called particles since, although possessing reverse transcriptase activity and high molecular weight RNA of 60-70S, they possess no apparent biological activity and are not released from the cell.

They are ~70 nm diameter spheres possessing a single outer membrane and an inner, doughnut-shaped, nucleoid.

Two types of A- particle have been distinguished. The first type bud into the cisternae of the endoplasmic reticulum and are known as intracisternal A- type particles (Dalton 1972 (a)). The second type remain in the cytoplasm of the cell and are named intracytoplasmic A- type particles (Dalton 1972 (a)).

The two types of particle are both designated A- type on the basis of their morphology and, more specifically, on their toroidal-shaped nucleoid. However, there are certain differences in their

morphologies which has been shown by work in the murine system (Wivel et al, 1975).

The intracisternal type (Kuff et al, 1972; Wivel et al, 1973) is surrounded by an outer shell of ~5nm in width, probably picked up during budding into the cisternal space of the endoplasmic reticulum, and is of overall diameter ranging from 70 to 100 nm. In contrast, the cytoplasmic located type (Smith and Wivel, 1972; 1973) possesses no such surrounding outer layer and has a smaller diameter of 75 nm.

Immunologically, the two types of particle possess antigenically distinct major structural proteins whilst sharing some antigenic determinants (Wivel et al, 1975).

Recent findings have shown that intracytoplasmic A- type particles are of similar genetic constitution, and share extensive sequence homology with the B- type virus as in the murine mammary tumour system (Michaelides et al, 1977). This, coupled with the evidence that the type -A particles in this host/virus complex are antigenically similar to the B- type virus, (Smith and Lee, 1975), supports the theory that type -A particles are the precursors to type -B viruses (De Guilli et al, 1975).

A- particles possess structural components which have been shown to be the precursors of the polypeptides in B- types by immunological experiments (Smith and Lee, 1975). These components may be a product of a cessation in the normal processing of precursor polypeptides of the B- type for some, as yet unknown, reason.

Evidence for A- types being involved in C- type maturation has been gathered using hybridization and immunological techniques, although results have been sometimes difficult to interpret (de Guilli et al, 1975).

Type -A particles have been reported to be present in placental tissues of various species of animal, and their presence has been reported in early mouse embryos (Biczysko et al, 1973; Chase & Piko, 1973). This incidence in developing tissues poses a question as to the rôle of the particles in normal development and gene expression.

B-Type Virus

The type-B RNA tumour viruses are most commonly found in association with mammary tumours and the milk of infected animals. A well-documented example of this type of virus is the murine mammary tumour virus (MMTV), (Lyons & Moore, 1965).

B-type particles are approximately 100 nm in diameter and have a buoyant density of 1.18 g/ml. They are enveloped, and protruding through this layer are many spikes, 5-10 nm in length (Calafat & Hageman, 1968). The nucleoid of the mature virus is of even electron density and is eccentrically located (Bernhard, 1958).

These viruses are antigenically distinct from type-C viruses in both their group-specific, gs, antigens and in their reverse transcriptase antigen (Nowinski et al, 1973; Toöze, 1973; Verma, 1977).

As stated in the previous section, it appears that intracytoplasmic type-A particles may sometimes become the nucleoids of type-B virus (Sarkar & Dion, 1975), but, irrespective of the source of this component of the virus, B-type nucleoids are assembled just below the plasma membrane of the cell. Thus the nucleoid of the B-type is almost completely formed, ultrastructurally, before the release of the virion from the cell, in contrast to type-C virus which assembles its nucleoid after release from the cell (de Harven, 1975).

After budding through the host cell membrane, the particle undergoes a period of maturation, the most readily observed ultrastructural change being the shift of the nucleoid from a central to an eccentric position, this shift being accompanied by an increase in electron density of the nucleoid to a more uniform appearance.

A distinction between 'mature' and 'immature' virus particles is

based on the state of condensation of the nucleoid - nucleoid collapse proceeding with maturation. It is by this criterion that particles destined to become type-B virus may be distinguished from those becoming type-C.

C-Type Virus

C-type RNA tumour viruses are enveloped viruses of 100 nm in diameter. They are of 1.16 g/ml buoyant density and possess a nucleoprotein core of 70-80 nm (Tooze, 1973). There are no spikes or projections on the virus membrane and the nucleoid is centrally located. Unlike B-type virus, the C-type nucleocapsid maturation takes place during and after budding. As assembly proceeds, the nucleocapsid develops from a crescent form into a toroidal one. After budding, the nucleoid becomes more electron dense. This morphological change is associated with biochemical changes within the virion (Sarkar et al, 1971).

C-type viruses of the murine and avian systems are the best characterized of the RNA tumour viruses. The structure and replication of these viruses will be described in detail later.

D-Type Virus

Mason-Pfizer monkey virus (MPMV) is the best described example of this virus type. First isolated from a spontaneous mammary tumour of a Rhesus monkey (Chopra & Mason, 1970), it bears similarities to both B- and C-type viruses (Kramarsky et al., 1971). Type D viruses have only been isolated from primates. MPMV morphogenesis is similar to that of B-type viruses. Two types of virus particle are present in MPMV infected cells. Inclusions of intracytoplasmic particles of 60-95 nm diameter are observed. These are putative viral nucleocapsids, their assembly being completed prior to budding.

The second particle type is extracellular, of 100-120 nm diameter, and 1.16 g/ml buoyant density (Schlom & Spiegelman, 1971). These particles possess smooth membranes, unlike B-type viruses, and pleomorphic nucleoids. Type D viruses may be also differentiated from C-type viruses by the immunological and biochemical properties of their internal non-glycosylated structural proteins (Parks et al., 1973; Tronick et al., 1974).

Host Range

Host range has been extensively studied in the avian and murine retrovirus systems. There are three classes of retrovirus in the murine leukemia virus system, as defined by their host range.

- a) The ecotropic viruses are endogenous mouse viruses in that they can be recovered from uninfected cells, and their proviral DNA is transmitted vertically via the germ line of the animal. They can infect cells of mouse and rat but not cells of other species.
- b) The xenotropic viruses are also endogenous viruses of the mouse. They cannot, however, infect mouse cells, only cells of other animal species such as rabbit, mink, rat and duck (Levy, 1973; Levy, 1975; Levy et al., 1975; Levy & Pincus, 1970).
- c) The third class of murine viruses are the amphotropic viruses (Hartley & Rowe, 1976), first isolated from wild mice. These viruses have the host range characteristics of the N-tropic (see below), ecotropic and xenotropic viruses. However, they differ in their interference and neutralization specificities.

Ecotropic and xenotropic virus types are also known in other mammalian species, eg cat and baboon. The ecotropic viruses may be further subdivided into N-, or B-, or N/B-tropic viruses. This classification is determined by the ability of the virus to replicate in NIH-Swiss (N-type) cells, BALB/c (B-type) cells or both (Hartley et al., 1970).

Two factors appear to determine the host range of the RNA tumour viruses (Gazdar et al., 1974):

- 1) a cell surface receptor which permits the attachment and entry of virus into the cell; and

ii) an intracellular restriction mechanism, eg the Fv-1 gene product.

It has been shown that the exclusion of viral entry is the basis for the eco-, xeno- and amphi- tropism of murine leukemia viruses (Besmer & Baltimore, 1977). Availability of specific viral receptors on the various cells is a key factor in determining viral host range.

The host range of certain avian viruses, eg ASV, is also determined by the availability of cellular receptors which permit infection of the cell. Susceptibility to infection in chickens is due to a dominant gene, specified by at least three loci (Weiss 1976).

When infection of mammalian cells is attempted with ASV, certain cells are non-permissive due to host restriction at the cell surface (Boettiger et al., 1975).

The intracellular restriction process has been shown, in mouse cells susceptible to infection by ecotropic MuLV, to be determined by the Fv-1 genetic locus (Pincus et al., 1971; Lilly & Pincus, 1972). Resistance to infection is a dominant factor, the Fv-1ⁿ allele determining resistance to B-tropic viruses and the Fv-1^b allele to viruses of N-tropism (Jolicoeur & Baltimore, 1976). The resistance is not absolute, eg high virus concentrations can overcome the restriction.

The site of the restriction of viral growth appears to be after entry of the virus into the cell and before viral integration. The process of viral DNA integration is prevented and levels of viral RNA within the cell are reduced. A cellular 'factor' is probably involved in the process and also a virion protein, a phenotypic property of the virus (Rein et al., 1976).

Certain strains of ALSV are restricted in their replication in some permissive cells by an intracellular mechanism (Linial & Neiman, 1976; Robinson, 1976). This restriction differs from the Fv-1 gene in that the restriction of growth is a recessive factor.

Structure of C-Type Retroviruses

Chemical Composition

The RNA tumour viruses have the overall chemical composition - 60-70% protein, 20-30% lipid, 2% carbohydrate and less than 2% RNA (Robinson & Duesberg, 1968; Tooze, 1973). A small amount of DNA has also been found within virions (Levinson et al, 1970; Biswal et al, 1971; Byers et al, 1979). It has been suggested that this DNA is of cellular origin (Varmus et al, 1971).

RNA

Virus RNA when extracted with phenol/SDS and sedimented in a neutral sucrose gradient gives peaks at 60-70S and 4-12S (Robinson et al, 1965). 60-70S RNA was determined to be single stranded by its susceptibility to RNase digestion, the dependence of its sedimentation rate on ionic strength, and the lack of complementary base pairing shown by its base composition (Robinson et al, 1967).

The smaller RNA component is partly degraded 60-70S RNA (Robinson et al, 1965), but also contains certain cellular tRNA's (Erikson & Erikson, 1970; Sawyer & Dahlberg, 1973). These included tRNA's appear to depend on the viral polymerase (Sawyer & Hanafusa, 1979), and their distribution is specific for a given virus grown on a particular cell type.

Mild denaturation of the 60-70S RNA by heating or treatment with dimethylsulfoxide yields 30-40S RNA (Duesberg 1968; Duesberg & Vogt, 1970), and tightly bound 4S RNA (Faras et al, 1973), see later. This pattern of denaturation suggests that the 60-70S RNA is a complex, multimeric molecule whose components are hydrogen bonded. The 30-40S RNA corresponds to the haploid genome with a molecular weight of $3-4 \times 10^6$ daltons (Duesberg 1968; Erikson 1969).

The 30-40S RNA bears a structural resemblance to cellular messenger RNA (mRNA). The 3' end is polyadenylated with about 200 adenylate residues (Lai & Duesberg, 1972; Wang & Duesberg, 1974), and the 5' end is capped by the structure $m^7G^{5'}ppp^{5'}NmpNp$ (Furuichi *et al.*, 1975; Rose *et al.*, 1976). It has been shown that the 30-40S RNA can act as an mRNA for the gag and pol (see later) gene products, in vitro and in vivo (von der Helm & Duesberg, 1975; Salden *et al.*, 1976) and is therefore of positive polarity. The RNA of ASV is also methylated internally (Beemon & Keith, 1976).

Oligonucleotide analysis has provided evidence to support the hypothesis that the 30-40S RNA molecules contain a complete set of viral genes. The genome of RNA tumour viruses has a unique genetic complexity of $3-4 \times 10^6$ daltons. This corresponds to a 30-40S molecule of a single-stranded RNA (Beemon *et al.*, 1976). This result also indicates that the 60-70S genomic RNA of the RNA tumour viruses is polyploid.

The comparative molecular weights of the 30-40S and 60-70S RNA species suggest a diploid genome and electron microscopic observations appear to confirm this (Bender & Davidson, 1976; Kung *et al.*, 1976).

By using relaxed circular SV40 DNA, tagged with poly (dT) tails by terminal transferase, the 3' poly A-containing ends of the 60-70S RNA were visualized (Bender & Davidson, 1976). A Y-shaped structure was observed in the centre of the molecule and was termed a dimer linkage. This structure is believed to join the 5' ends of two 30-40S molecules, the 3' poly A-containing ends being distal to the linkage. The 60-70S molecule therefore has 3'-5' polarity running in opposite directions in the two halves of the molecule. The assembly of the 60-70S genomic RNA complex from its 30-40S RNA components has not been reported.

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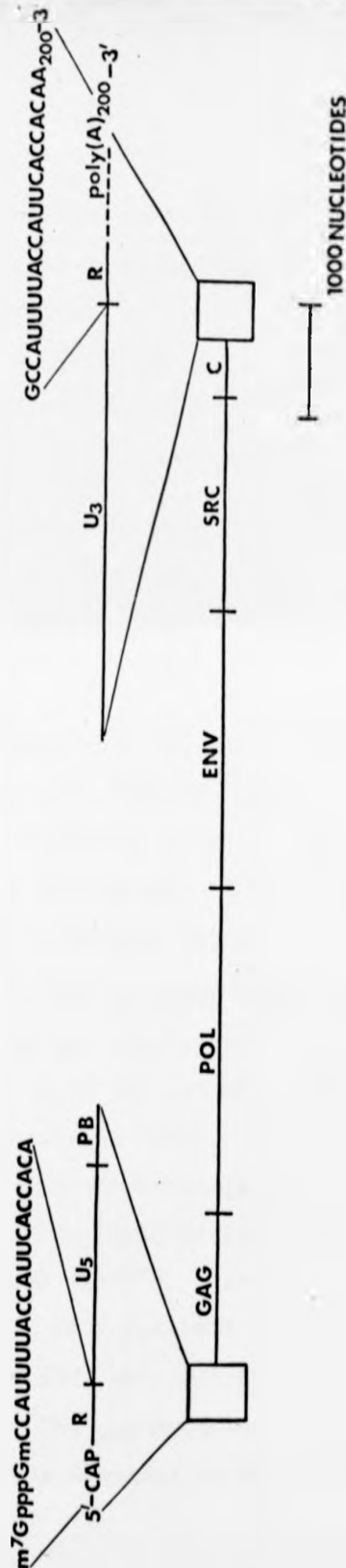
This is consistent with the theory that the dimer linkage is caused by base-pairing. This may involve either a small linker RNA molecule or regions of self-complementarity.

The finding that 30-40S subunits of ASV (Haseltine et al, 1977; Schwartz et al, 1977), AMV (Stoll et al, 1977) and MuLV (Coffin et al, 1978) are terminally redundant supports the latter hypothesis, although these sequences are not in the postulated position in the molecule.

The terminally redundant sequences of various strains of ASV, of both endogenous and exogenous viruses, bear similarities (Coffin, 1979). The terminal redundancies are involved in viral replication and will be discussed below.

Non-defective RNA tumour viruses contain at least three genes: gag, pol and env. One or more of these genes may be absent in the replication-defective sarcoma viruses. However, they possess additional genetic material. In ASV, this material is called src, (see later). A small region, c, is also found in some viruses (Wang et al 1975; Tal et al, 1977).

Figure 1 illustrates the gene organization of the ASV genome (Coffin, 1979). There is provisional evidence that the gene order in MuLV/MSV is identical to that of ASV (Parks et al, 1976). The src gene has been mapped close to the 3' terminus of the genome (Hu et al, 1977), and it may be deduced from vRNA translation data that gag and pol are located in the same positions as in the ASV genome (Philipson et al, 1978; van Zaane & Bloemers, 1978).



R : Redundant sequence 16-21 nucleotides
 U₅ : Unique 5' sequence 80 nucleotides
 U₃ : Unique 3' sequence 200 nucleotides
 PB: Primer Binding site (see later)

FIGURE 1

Structure of the non-defective ASV genome as a representative RNA virus.

ASV is unusual in that src is present, but the virus is replication competent. The terminal redundancy illustrated is that of the Prague-C ASV genome. Gene boundaries are approximate, however, the gene order is certain 5'-gag-pol-env-src-c-3'. The terminal redundancy in MoMLV is longer, 60 nucleotides, and has no sequence or structural relationship to any of the various ASV strain sequences (Coffin *et al.*, 1978).

(from Coffin, 1979)

Proteins

The nomenclature used for the proteins of the RNA tumour viruses is that proposed by August et al., (1974), and is as outlined below:

- p : protein
- pp : phosphoprotein
- gp : glycoprotein
- Pr^{sup} : precursor - superscript denotes viral gene encoding the precursor

These prefixes are followed by the first two digits of the molecular weight of the protein ($\times 10^{-3}$).

gag

The gag gene codes for the non-glycosylated internal structural proteins of the virion. gag stands for group specific or gs antigens. The major antigenic sites of these proteins are shared amongst viruses of a group or species. The antigens shared between one viral species are known as species specific.

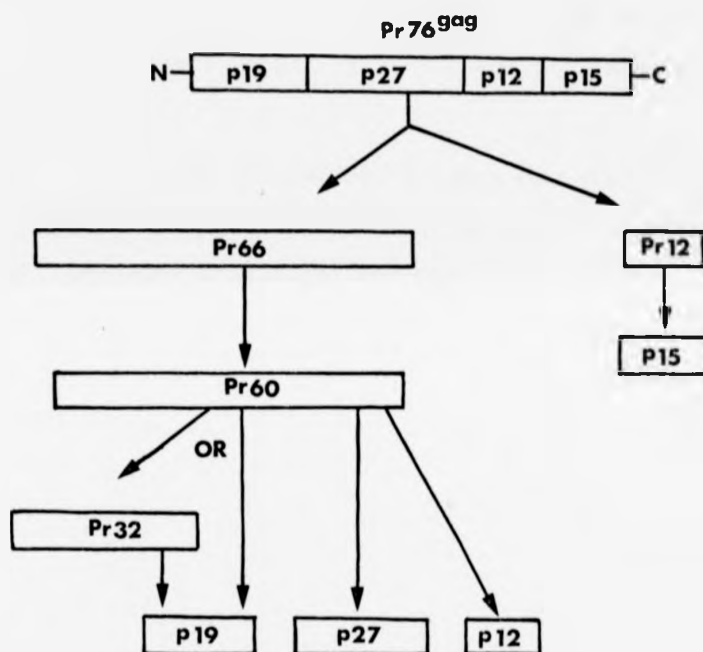
In the ALV, the gag gene codes for four proteins p27, p19, p15 and p12; in the MLV, the proteins are p30, p15, p12 and p10. A polyprotein precursor of 76,000 daltons (Pr76^{gag}) is found in the avian system (Vogt & Eisenman, 1973). A larger precursor, Pr180^{gag pol} has also been observed (Opperman et al., 1977; Hayman, 1978). This protein arises from the uninterrupted translation of the gag and pol genes and includes Pr76^{gag}. Cleavage of Pr76^{gag} gives rise to the intermediates Pr66, Pr60, Pr32 and Pr12 in infected cells and these are then further cleaved to p27, p19, p15 and p12 (Vogt et al., 1975; Weiss et al., 1977). Figure 2. The gag protein p15 of ASV is a protease (von der Helm, 1977) and is involved in the cleavage of Pr76^{gag}. In vitro cleavage

FIGURE 2. Processing of the avian virus gag precursor Pr76.

Pr76^{gag} is first cleaved to remove Pr12 from the C-terminus.

Pr12 is modified to become the protease p15 which is responsible for the cleavage of the other gag gene products.

p19 is phosphorylated (Lai, 1976).



of Pr76^{gag} by p15 results in the sequential appearance of p15, p27 and p19 (Moelling et al., 1980). This p15 will also cleave the Pr65^{gag} of FeLV into p30, p15 and p10 (Khan & Stephenson, 1979).

The gag precursors in MLV infected cells are Pr80^{gag} and Pr65^{gag} (van Zaane et al., 1976), although recent evidence indicates that Pr80^{gag} is glycosylated (Edwards & Fan, 1979). Cleavage of Pr65^{gag} results in the appearance of p15, p12, p30 and p10 (Barbacid et al., 1976), Figure 3.

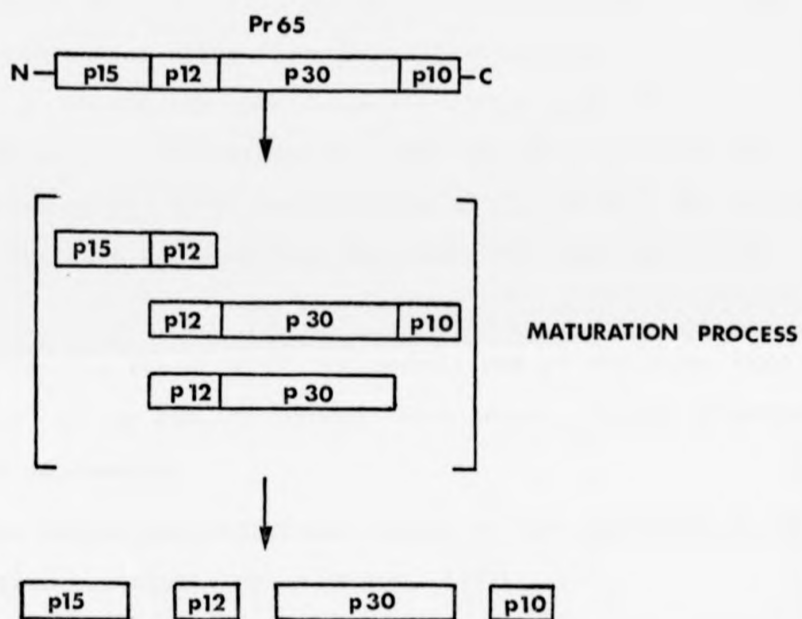
Large polyproteins of 150,000-200,000 daltons can also be found in infected cells of the murine system (Naso et al., 1975) and represent 'read-through' products of the gag and pol genes (see later).

FIGURE 3. Sequence and maturation process of the gag gene product of mammalian C-type viruses.

Details of the events in the maturation process are, as yet, unknown.

p12 is phosphorylated (Pal et al, 1975).

(from Barbacid et al, 1976)



RNA tumour viruses carry within the virion a virus-coded RNA-dependent DNA polymerase known as Reverse Transcriptase (Baltimore, 1970; Temin & Mizutani, 1970), (EC 2.7.7.7). The enzyme possesses RNA- and DNA-dependent DNA polymerase activities (Temin & Baltimore, 1972; Verma 1977).

An RNA.DNA hybrid-specific RNase activity is also associated with reverse transcriptase. This activity is known as RNase H (Moelling et al, 1971; Sarngadharan et al, 1976). The enzyme is an exo-ribonuclease and requires free ends (Baltimore & Smoler, 1972).

Reverse transcriptase transcribes the genomic RNA of the virus into a DNA 'provirus' via an RNA.DNA hybrid, (see later), and is essential for viral gene expression.

RNase H and DNA polymerase activities reside on the same polypeptide but have different catalytic sites (Verma, 1977).

The structures of purified avian and murine reverse transcriptases are markedly different.

The avian enzyme has a sedimentation coefficient of 7.5S (Grandgenett et al, 1973; Verma et al, 1974) and a molecular weight of 170,000 daltons. The holoenzyme consists of two types of polypeptide - one of 95,000 daltons, the β subunit, and one of 65,000 daltons, the α subunit (Kacian et al, 1971; Gibson & Verma, 1974). The α subunit is derived from the β subunit by proteolytic cleavage (Verma, 1977; Moelling, 1976). A recent report indicates that p15 may cleave the β subunit into α subunits in the avian system (Moelling et al, 1980). β subunits may be phosphorylated (Hizi & Joklik, 1977).

The murine reverse transcriptase has a sedimentation coefficient of 4-4.5S on glycerol gradients (Moelling, 1974; Verma, 1975), only

one polypeptide has been observed of 70-80,000 daltons. The polypeptide resembles the β subunit of the avian enzyme in its template affinity and RNase H activity (Moelling, 1976).

The DNA polymerase activity may be characterized by the utilization of various template-primer combinations (Verma, 1977), and these will be discussed later.

Large polypeptides of 200,000 daltons in the murine system (Jamjoom et al., 1977) and 180,000 daltons in the avian system (Opperman et al., 1977) are precipitated by antisera to the gag proteins. The large polyprotein can also be precipitated by antisera to the purified reverse transcriptase (Jamjoom et al., 1977; Opperman et al., 1977). Evidence supporting the hypothesis that reverse transcriptase is synthesized as a gag-pol polyprotein comes from the use of temperature-sensitive mutants (Vogt & Hu, 1977), observations on messenger RNA (Hayward, 1977; Weiss et al., 1977) and cell-free translation experiments (Paterson et al., 1977).

There are less than 100 molecules of reverse transcriptase per virion (Panet et al., 1975), 20-50 fold lower than the gag gene products. Thus the two gene products must be under different controls. The nature of these controls, the mechanism of formation of the Pr^{gag-pol} polyprotein and the fate of the gag portion of the polyprotein after pol product formation are unknown. It is known however that an addition of Yeast amber suppressor tRNA to an in vitro translation system primed with Rauscher MLV RNA increases yield of Pr180^{gag-pol} relative to Pr78^{gag} (Philipson et al., 1978) suggesting a gag.pol read through model.

The reverse transcriptase plays a vital rôle in the replication of the RNA tumour viruses and its mode of action will be discussed in the Replication section.

env

The env gene codes for the envelope proteins of the RNA tumour viruses. In the avian system, the two major envelope proteins are of 85,000 and 37,000 daltons molecular weight. Both are glycosylated and are known as gp85 and gp37 (August et al, 1974).

A third, non-glycosylated protein, p10, may also be located in the envelope. p10 is not contained within the gag precursor Pr76 (Vogt et al, 1975).

The two glycoproteins are localized in structures on the surface of the virus particles as knobs and spikes (Rifkin & Compans, 1971; Nermut et al, 1972). Gp85 forms the knob structure, gp37 the spike (Bolognesi et al, 1972) and both are linked, as dimers, by disulfide bonds (Leamnson & Halpen, 1976).

In the murine leukemia virus, the major envelope glycoprotein is gp70. A nonglycosylated protein of 15,000 daltons also present in the envelope of MLV is termed p15E to distinguish it from p15 core protein (Ikeda et al, 1975). Gp70 and p15E are also linked, as dimers, by disulfide bonds (Leamnson et al, 1976; Witte et al, 1977).

A smaller protein p12 E has also been described and appears to be a breakdown product of p15 E (Naso et al, 1976; Famulari et al, 1976). p10 may be derived from gp37 in the same manner.

In the avian system, gp85 and gp37 are derived by proteolytic cleavage from a polyprotein precursor gPr92 (Moelling & Hayami, 1977; Klemenz & Diggelmann, 1978). This cleavage takes place extracellularly (Klemenz & Diggelmann, 1979). gPr92 may be formed by the glycosylation of a 62-64,000 dalton protein (Diggelmann, 1979; Pawson et al, 1980) which is probably the primary product of the env gene.

A high molecular weight precursor of 82-90,000 daltons has been found in the murine system (van Zaane et al, 1975; Famulari et al, 1976) which is processed into gp70 and p15E (Naso et al, 1976). A 70,000 dalton protein has also been observed in cells in which glycosylation is inhibited (Shapiro et al, 1976), although no conversion of this protein to gPr90 or gp70 was observed.

The envelope glycoproteins determine the type-specific properties of the RNA tumour viruses, eg interaction with neutralizing antibodies and viral interference (Hunsmann et al, 1974; Steeves et al, 1974). They also determine the host-range of the viruses via the envelope spike structures (Weiss, 1976). The function of the spikes is to mediate adsorption and penetration of the virus into susceptible host cells by interaction with cellular receptors (Bauer, 1974). Enzymic removal of the spikes results in a loss of infectivity (Rifkin & Compans, 1971).

Transforming Genes

Several transforming genes have been discovered in the Retroviridae. The four best documented are outlined in Table 2.

These transforming genes are genetically distinct from each other. It has recently been shown (R J Avery, personal communication), that many of the transforming genes of mouse, rat, feline primate and avian viruses are distinct from one another. A new nomenclature has been formulated, eg transforming genes of rat sarcoma viruses, Harvey, Kirsten and Rasheed have been named RAS - these three viral genes being related to each other but distinct from the transforming genes of other sarcoma viruses. Other examples are shown in Table 2.

The product of a transforming gene has been best characterized in the avian system and the description which follows refers to the avian sarcoma virus, unless otherwise stated.

ASV causes sarcomas in birds and can cause morphological transformation in both avian and mammalian fibroblasts. The gene, designated src for sarcoma gene, is responsible for this neoplastic transformation (Hanafusa, 1977).

The polypeptide product of the src gene is a 60,000 dalton phosphoprotein (Brugge & Erikson, 1977; Purchio et al, 1978; Levinson et al, 1978; Collett et al, 1979), pp60^{src}. A protein kinase activity has been shown to be associated with this polypeptide (Collett & Erikson, 1978; Levinson et al, 1978; Sefton et al, 1979). The kinase donates the terminal phosphate from ATP or GTP to an acceptor protein. The results of Levinson et al (1978) and Sefton et al (1979) suggest that the protein kinase activity is a product of the src gene and not a contaminating, cellular protein fortuitously associating with pp60^{src}. Recent results indicate that the protein kinase activity is necessary for cell transformation (Owada & Moelling, 1980; Sefton et al, 1980).

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TABLE 2

Four transforming genes of the RNA tumour viruses

<u>GENE</u>	<u>VIRUS</u>	<u>REFERENCE</u>
src	ASV	Hu <u>et al.</u> , 1977
erb	AEV	Roussel <u>et al.</u> , 1979
mac	MC29	
myb	AMV	Beug <u>et al.</u> , 1979

The cellular target phosphorylated by the protein kinase is unknown. One substrate for the enzyme may be the cytoskeleton (Weber et al., 1974; Ash et al., 1976; Rohrschneider, 1979; Wang & Goldberg, 1979). In vitro translation data suggests that the src gene product is a protein of 62,000 daltons (Karmine & Buchanan, 1978), and that a 2000 dalton segment is lost forming pp60^{src}. It is postulated that the 2000 dalton segment is a signal sequence for the insertion of pp60^{src} into cellular membranes.

Origin of the Transforming Genes

By using radioactive probes complementary to the src gene (Stehelin et al., 1976 (a)), it was shown that the DNAs of uninfected avian cells contained cellular sequences related to src (Stehelin et al., 1976 (b)). These cellular sequences, designated sarc, have been highly conserved during avian evolution. Cellular sarc in avian cells is transcribed into RNA (Spector et al., 1978 (a) & (b)), although its function in both normal and neoplastic cells is unknown.

ASV is replication competent, unlike the murine sarcoma viruses, which require a helper leukemia virus. Most strains of MSV were obtained by passage of leukemia viruses through rats or mice. Hybridization studies have suggested that sequences responsible for cell transformation were picked up from host cells with the loss of viral replicative genes. This may have occurred by recombination during passage of the leukemia virus through the host (Scolnick et al., 1973; Scolnick & Parks, 1974; Andersson & Robbins, 1976; Hanafusa, 1977). Consistent with this, HaSV and KMSV, isolated after passage of leukemia virus through rat, possess rat specific sequences. Moloney sarcoma virus, isolated from mice after passage of leukemia virus, possess mouse specific sequences.

These results, in both avian and murine systems, suggest a cellular origin for src and other transforming genes. It has not been possible, however, to demonstrate the formation of a transforming virus by passage of a leukemia virus in the avian system.

Strains of ALSV share a sequence of about 1000 nucleotides at their 3' end of the genome (Tal et al, 1977). This sequence has been denoted the c, for common, region (Wang et al, 1975; 1976). c is conserved even when deletions in src occur (Tal et al, 1977), and its presence confers a selective growth advantage to some viruses (Tsichilis & Coffin, 1980). These findings coupled with the observation that the c region is repeated at the left end of proviral DNA, outside of the RNA transcription region (Hsu et al, 1978; Shank et al, 1978), suggest a control function for c (Tsichlis & Coffin, 1980).

c may have its own mRNA and gene product (Purchio et al, 1977), a protein of 29,000 daltons. The function of this protein is unknown.

It has recently been reported that a conserved region of RNA also exists in mammalian viruses, between 50 and 400 nucleotides from the 3' terminus of the genomic RNA (Kominami & Hatanaka, 1979).

mRNAs

The protein biosynthesis of both mammalian and avian viruses is very very similar. The major internal proteins are contained within a polyprotein precursor translated from genomic size mRNA. This precursor is subsequently cleaved to yield the mature gag proteins.

The reverse transcriptase is also synthesized from a full-length genomic mRNA. Its polyprotein precursor appears to be a gag-pol 'read-through' product which is processed to form the enzyme.

Envelope glycoproteins are translated from a subgenomic 22S mRNA in the murine system, 28S mRNA in the avian. The primary env gene products are glycosylated polyproteins of 80,000-92,000 daltons, although a smaller, unglycosylated, protein has been found in the avian system. These precursors are transported to the cell plasma membrane and are processed extracellularly, or during virus budding, into the mature env gene products.

The src gene product in ALSV is translated from a 21S mRNA. One product of this gene is a 60,000 dalton protein -the existence of other protein products is unknown.

The c 'gene' may have its own mRNA of 12S and gene product of 29,000 daltons (Purchio et al, 1977; Krzyzek et al, 1978). However, these results are tentative.

In the ASV system, it has been shown that 5' terminal nucleotide sequences from the virus genome are transposed to the 5' termini of smaller viral mRNAs (Cordell et al, 1978; Krzyzek et al, 1978).

At least 104 nucleotides are transposed or 'spliced' onto each viral mRNA in infected cells by some unknown mechanism. The function of the spliced sequences is also unknown. Patterns of protein biosynthesis for the avian and murine systems are summarized in Figures 4 & 5, adapted from Weiss et al, (1977) and van Zaane and Bloemers, (1978).

FIGURE 4. Biosynthesis of ALSV proteins : Transcription and Translation.

Diagram represents a summary of the available data on all ALSV strains. [] is still tentative.

■ = leader sequences from 5' end.

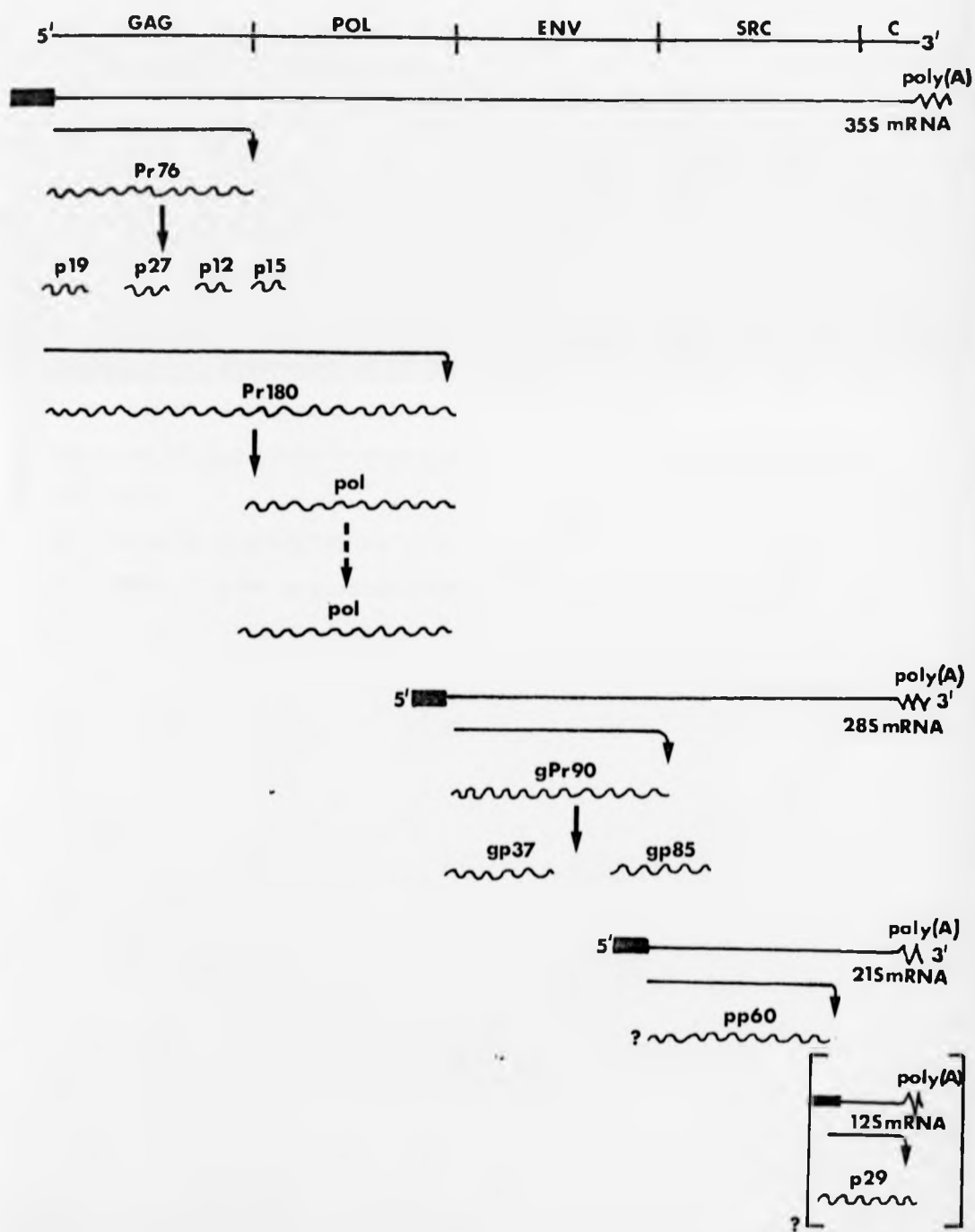


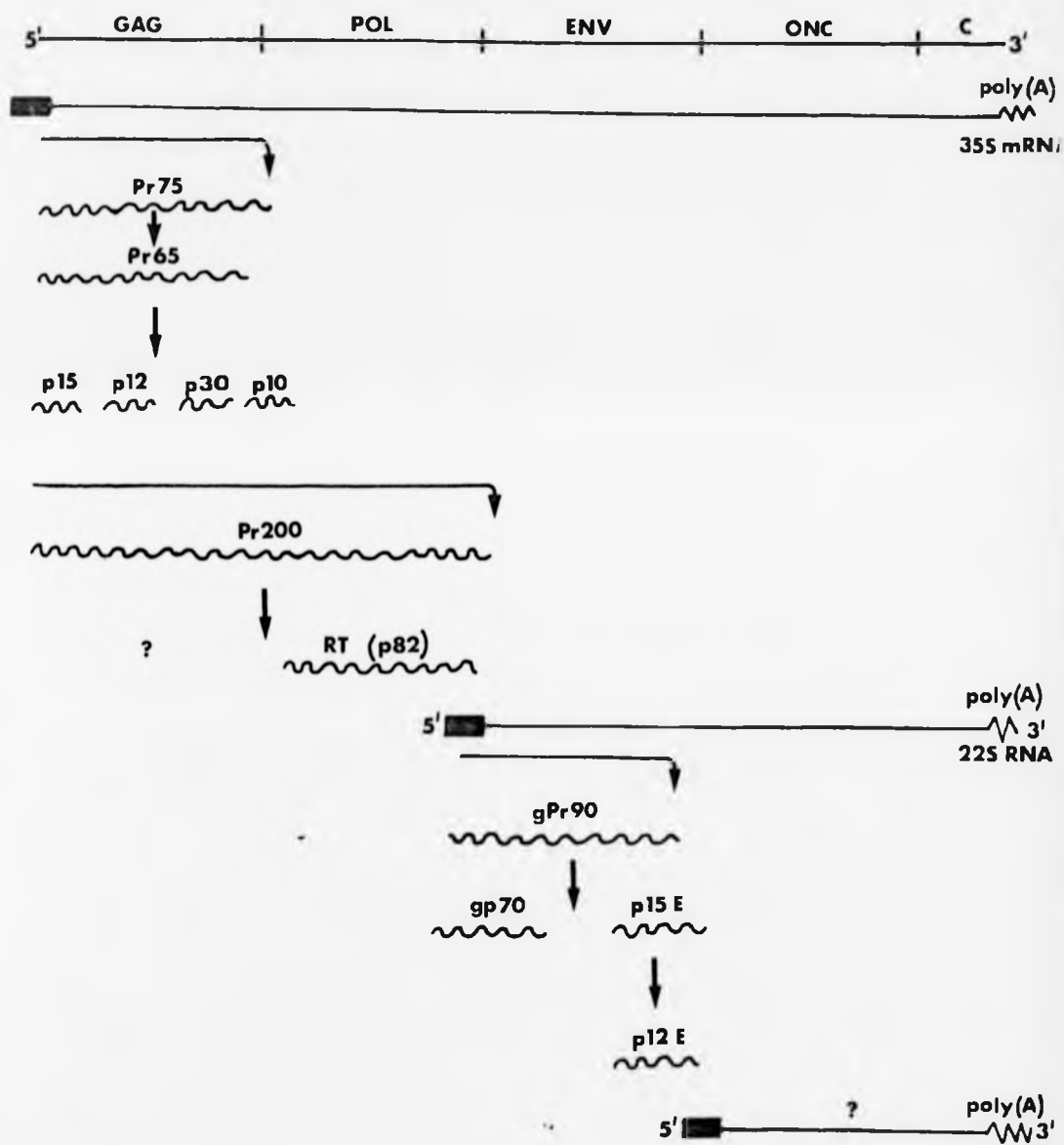
FIGURE 5. Biosynthesis of MLV proteins.

Diagram represents data mainly from Rauscher MLV.

Presence of onc, putative neoplasia inducing gene, is hypothetical - see text.

RT : Reverse Transcriptase

■ = leader sequences from 5' end.



Lipids

Virion lipids are contained within the viral envelope. The lipid composition of the virus reflects the lipid composition of the host cell plasma membrane (Quigley et al, 1971; 1972). This is expected since the virus buds from the cell. There are slight differences in certain phospholipids - however, these may be due to selective contamination of virions with non-envelope host cell lipids.

Structure and Organisation of the Virion

A diagrammatic representation of a typical Type-C retrovirus particle appears in Figure 6 (Vogt & Hu, 1977; Bishop, 1978; Bolognesi et al., 1978). In the following discussion the avian system is used as a model. Figures in brackets represent proteins of the murine system.

The envelope of the retroviruses is derived from the host cell plasma membrane, see earlier. Spikes protrude through the viral envelope and are composed of the major envelope glycoproteins, gp35 and gp85, (gp70; p15E). The env gene-coded protein p10, (p12E) , is also associated with the envelope.

Within the envelope is an inner coat surrounding a core shell. The inner coat is composed of the phosphoprotein pp19 (pp12).

The core structure may be obtained by treating intact virions with detergents or other treatments to remove the viral envelope (Bader et al., 1970; Stromberg, 1972) - the major core protein being p27, (p30).

The core contains a ribonucleoprotein complex of RNA, reverse transcriptase and two other gag coded proteins, (see Figure 6), that bind to the virion RNA.

The exact location of p15 in either avian or murine systems is unknown.

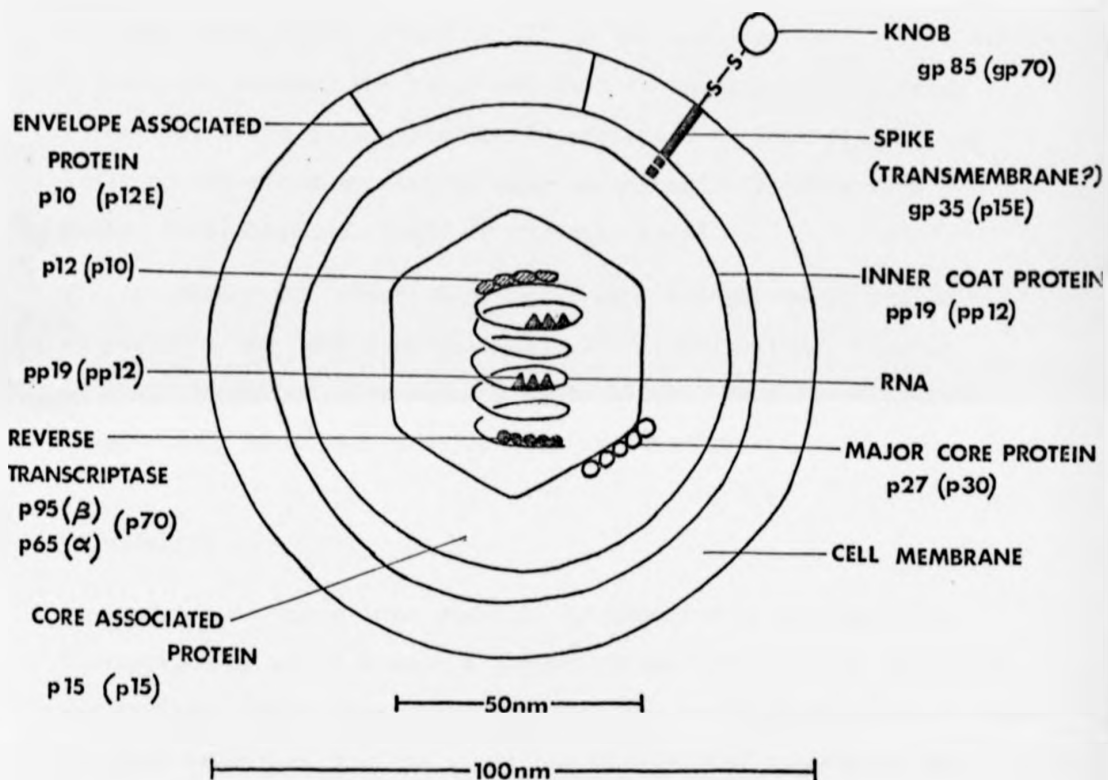


FIGURE 6. Diagrammatic representation of a Retrovirus Type-C Particle.

The localisation of avian and murine, (bracketed), proteins is based on data presented by Vogt and Hu, 1977; Bishop, 1978; and Bolognesi *et al*, 1978.

Replication of Retroviruses

Attachment

The virus must first attach itself to the cell membrane. This initial interaction between the virus and cell is insufficient to cause infection, since cells genetically resistant to some viruses may still adsorb virus equally as well as susceptible cells (Steck & Rubin, 1966; Piraino, 1967; Crittenden, 1968).

The efficiency of infection of cells can be improved by adding polycations, eg DEAE-dextran (Vogt, 1967); polyanions, however, inhibit attachment (Toyoshima & Vogt, 1969). Thus electrostatic interactions must play a major rôle in attachment.

Penetration

Penetration of virus into the cell is mediated by the specific interaction of viral envelope glycoproteins and cellular receptors, see earlier. When viral glycoproteins are produced by the host cell, the cell receptors for the virus are blocked and subsequent re-infection by the same virus is prevented.

Uncoating

The mechanism by which the retroviruses are uncoated within the cell is unknown. There are three possible mechanisms:

- a) Phagocytosis - The virus may be taken up within a cellular vesicle. Viral and vesicular membranes coalesce and the viral core is released into the cytoplasm.
- b) Viral and cell plasma membranes may coalesce upon infection and the viral core immediately released into the cytoplasm.

- c) Penetration by the complete virion and subsequent removal of the envelope within the cytoplasm.

Evidence for all these possibilities has been obtained (Miyamoto & Gilden, 1971).

Transcription 1

Once the virus has penetrated the cell and has been uncoated, the virion RNA is transcribed by reverse transcriptase into virus-specific DNA (Varmus et al, 1974; Sveda et al, 1974; 1976; Leis et al, 1975).

Transcription begins near the 5' end of the genome, the primer attachment site in ALSV being between 103 and 118 residues from the 5' end (Taylor & Illmensee, 1975; Haseltine et al, 1977; Shine et al, 1977). The primers for DNA synthesis are cellular tRNAs (Taylor, 1977). In ALSV, the primer is tRNA^{Trp}, in murine leukemia virus tRNA^{Pro} (Harada et al, 1975; Dahlberg et al, 1975; Harada et al, 1979). The primer tRNA is more tightly bound to the 30-40S genome than the other genome associated 4S RNA species (Faras et al, 1973; Waters et al, 1975).

Polymerization of DNA is initiated on the 3'-OH terminus of the tRNA primer and proceeds towards the 5' end of the template, terminating near the cap structure. The reverse transcriptase therefore reaches the end of the 30-40S molecule before copying the entire genome. The short DNA molecule synthesized is termed 'strong stop' DNA (Coffin & Haseltine, 1977). The length of strong stop DNA varies between virus isolates (Haseltine & Kleid, 1978), eg 101 nucleotides in ALSV, 135 nucleotides in MoMLV.

Since DNA synthesis is initiated at the 5' end of the genome, a

mechanism must exist for the 3' end of the genome to be synthesized. It is postulated that this mechanism makes use of the terminal redundancy of the Retrovirus genome.

Reverse transcription starts at the tRNA primer on the genomic RNA. DNA is synthesized up to the 5' end of the genome, where it is displaced from the genomic template RNA, possibly by the RNase H activity associated with reverse transcriptase. The complementary copy of the 5' terminal repeat then hybridizes with the repeated sequence at the 3' end of the same RNA molecule or another 30-40S vRNA molecule. DNA is then synthesized 5'-3' up to the tRNA primer at the 5' end of the RNA, resulting in a full-length DNA copy of the genomic RNA.

Evidence supporting these models was presented by Junghans et al, (1977), and Haseltine et al, (1979).

The initial single-stranded DNA product is called minus (-) DNA, the genomic RNA, positive (+) RNA. A positive DNA strand is synthesized using the minus strand DNA as template producing a linear, double-stranded vDNA molecule (Varmus et al, 1976). Synthesis of the plus DNA strand is initiated on the minus strand before the latter is completed (Varmus et al, 1978).

Linear duplex vDNA is found mainly in the infected cell cytoplasm, although a small amount can be found in the nucleus.

Closed circular DNA duplexes are synthesized from these linear duplexes by some unknown mechanism (Shank & Varmus, 1978). These molecules are found in the nucleus of the cell 5-12 hours post infection (Gruntaka et al, 1976). Both linear and closed circular DNAs are infectious (Smotkin et al, 1975; Fritsch & Temin, 1977).

A model for the transcription of retroviral RNA is presented in Figure 7 (Coffin, 1979; Gilboa et al., 1979).

The structures of the linear proviral DNA found before and after integration are shown in Figure 8.

Legend to Figure 7

A model for the transcription of Retroviral RNA

- 1) Reverse transcriptase, (R), initiates DNA synthesis on a tRNA primer near the 5' end of a 30-40S RNA subunit. Polymerization continues to the 5' terminus synthesizing 'strong-stop' DNA, which includes complementary sequences to the 5' unique sequences, U₅, and the terminally redundant 5' sequence, T₅. These cDNA sequences are termed U'₅, and T'₅, (2).
- 3) Sequences of the vRNA which are in a DNA.RNA hybrid are removed, possibly by the action of RNase H.
- 4) The single-stranded DNA molecule formed in (3) may now associate with the 3' end of the same or a different 30-40S RNA subunit and the reverse transcriptase may proceed to copy the virion RNA into DNA through T₃, and U₃.

Several aspects of vRNA transcription after this point are in contention.

A detailed model of reverse transcription is presented by Coffin (1979), and Gilboa et al (1979).

FIGURE 7.

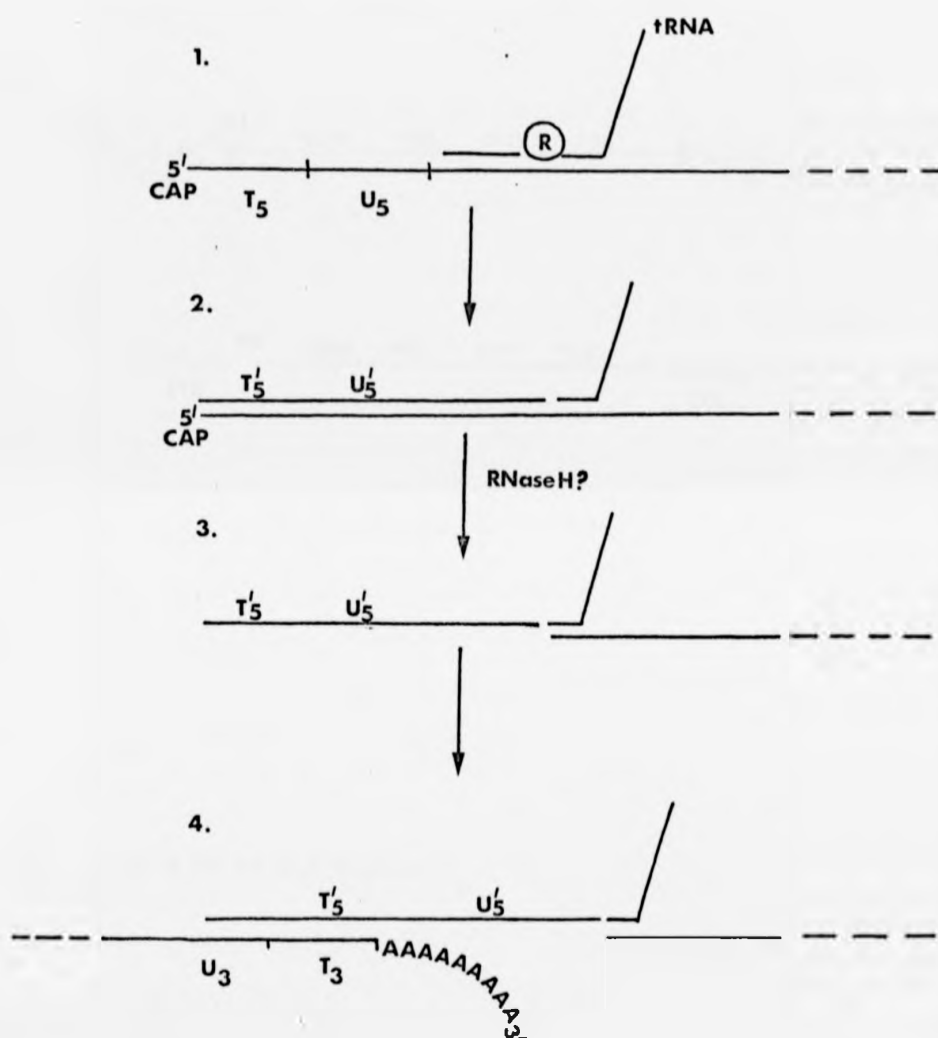
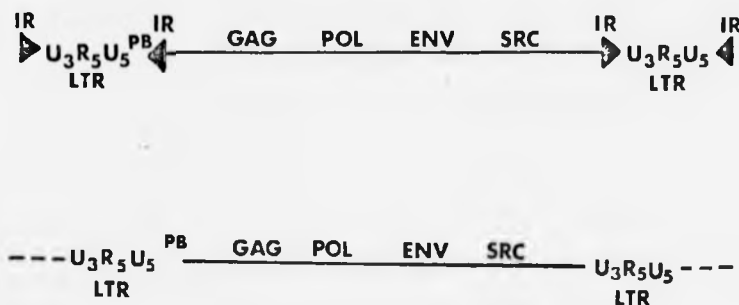


FIGURE 8



U_3 , R_5 , U_5 and PB as for Figure 7

FIGURE 8. Structures of ASV proviral DNA

- Unintegrated linear proviral DNA. Long terminal repeats, LTR, of U_3R_5 and U_5 (500-600 bp) are flanked by small inverted repeats, IR, of 5 bp. These are presumably present to aid in circle formation.
- Integrated ASV provirus.

(Coffin, 1979; Dr R J Avery, personal communication)

Integration

A small proportion of the vDNA molecules ultimately become integrated into the host cell DNA (Hughes et al, 1978), the number of integrated copies per infected cell being small (Varmus et al, 1973). Integration cannot take place without cell division and is an obligatory step in the viral life cycle, necessary for virus replication and cell transformation when this occurs.

It is not known which of the vDNA forms is the precursor to the integrated 'provirus', however, the most likely candidate is the closed circular form (Shank & Varmus, 1978; Coffin, 1979).

Evidence indicates that there is no unique site of integration within the cellular DNA of a cell population.

Many questions are still to be answered about the integration event, eg, what effect does the cellular integration site have on the transcription or stability of integrated vDNA? Is the site of integration important in cell transformation?

Transcription II

Transcription of the integrated provirus into mRNA is carried out by cellular RNA polymerase II (Jacquet et al, 1974; Rymo et al, 1974; Dinowitz, 1975), and in the ASV system is very efficient (Taylor, 1979). Rate of synthesis of mRNA from vDNA is independent of cell cycle, (Humphries & Coffin, 1976). Three species of polyadenylated cytoplasmic RNA have been found in cells infected with ASV; 38S, 28S and 21S (Hayward, 1977; Weiss et al, 1977). All three species may be translated into proteins, a portion of the 38S RNA representing the

pool of genomic RNA to be included in progeny virus. No 70S RNA is found within infected cells (Cheung et al, 1972).

All three mRNA species contain sequences homologous to those at the extreme 5' end of the ASV genome (Weiss et al, 1977), not present adjacent to these gene sequences in genomic RNA. The origin of these nucleotides may be from 'splicing' 5' sequences from the 30-40S RNA onto the mRNAs (Cordell et al, 1978; Krzyzek et al, 1978). The function of the 5' sequences on the mRNA is unknown.

Translation

Translation of virion mRNA utilizes the host cell protein synthesizing system. All the virion gene products undergo post-translational modifications, cleavage, phosphorylation, glycosylation etc., (Eisenmann & Vogt, 1978). (See Proteins section).

Budding

Virus is released by budding through the plasma cell membrane. Viral envelope glycoproteins migrate to the cell surface and are inserted at sites of virus budding. These glycoproteins interact with the viral cores, containing 30-40S RNA and the gag and pol gene products, which accumulate below the cell membrane at the budding sites. Bolognesi et al (1978) have postulated that the precursors to the internal structural proteins are associated by one end of their molecule with the envelope glycoproteins at the budding site. The other end is associated with the virion RNA.

After budding, the 70S RNA is produced and final processing of precursors into mature virion proteins takes place (Bishop, 1978;

Eisenmann & Vogt, 1978).

Virus particles may be assembled without envelope glycoproteins (de Guilli et al., 1975; Schwartz et al., 1976), reverse transcriptase, (Hanafusa et al., 1972) or viral RNA (Levin et al., 1974; 1976).

Summary

The life cycle of the RNA tumour viruses is shown in Figure 9.

Virus particles attach to the cell surface by a non-specific mechanism 1. A specific interaction then takes place between cell surface receptors and viral envelope ultimately resulting in the intracellular appearance of viral cores 2. Viral RNA is then transcribed into linear dsDNA via reverse transcriptase through a DNA.RNA intermediate 3. A fraction of this linear dsDNA becomes circularized by some unknown mechanism and can be isolated from the nucleus of the infected cell 4.

One or more copies of this proviral DNA are integrated into the host cell chromosomal DNA 5. Transcription of this DNA provirus is then carried out by cellular RNA polymerase II and viral mRNA can be found in the cell cytoplasm.

The mRNAs are translated into proteins which encapsidate 35S RNA as they are transported to the cell surface 7 and 8.

The maturing virions then bud from the cell, acquiring an outer envelope 9. Further maturation then occurs in the extracellular particles 10.

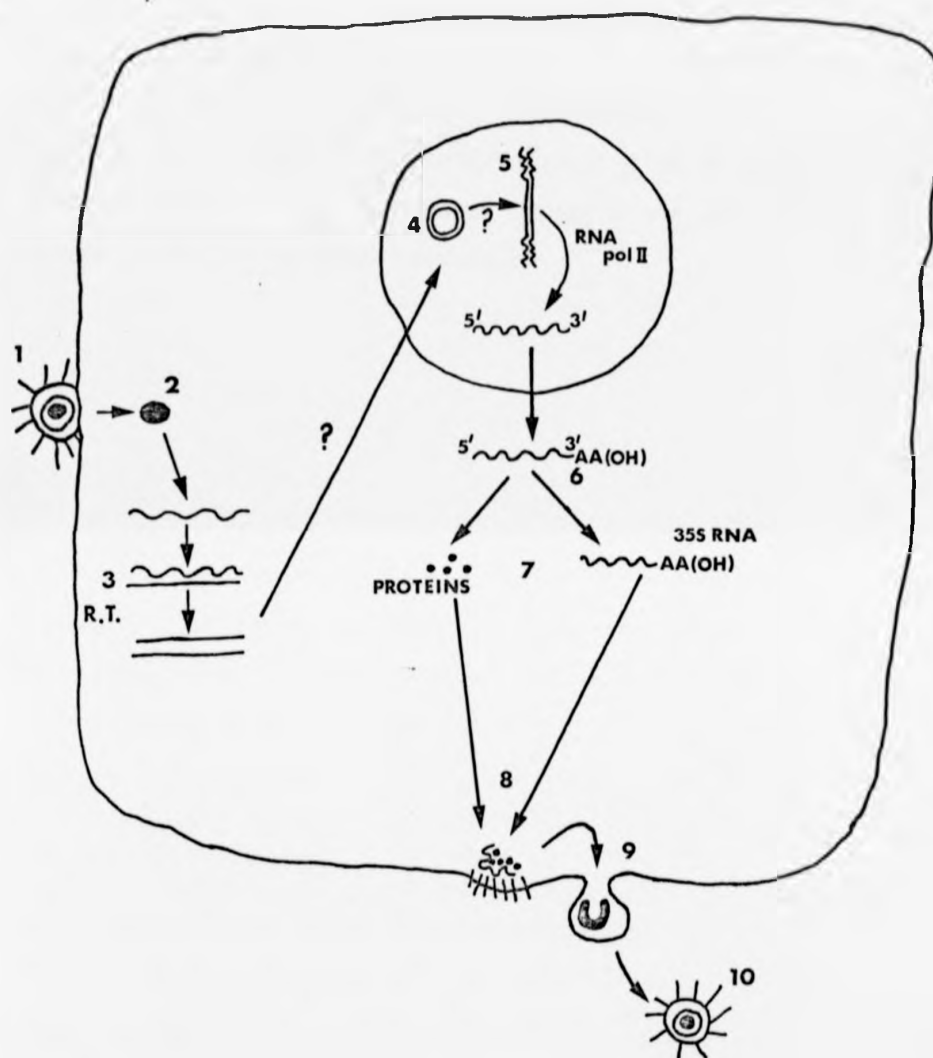


FIGURE 9. Life Cycle of RNA Tumour Viruses

R.T. : Reverse Transcriptase

(Adapted from Verma, 1977).

effects being observed upon subsequent culture of cells subjected to this procedure.

Temperature range for cell growth is the main difference between vertebrate and invertebrate cells. Vertebrate cells grown within temperatures of 32-40°C while invertebrates grow at temperatures between 16°C and 32°C. This point is important and will be discussed later in the Results Section.

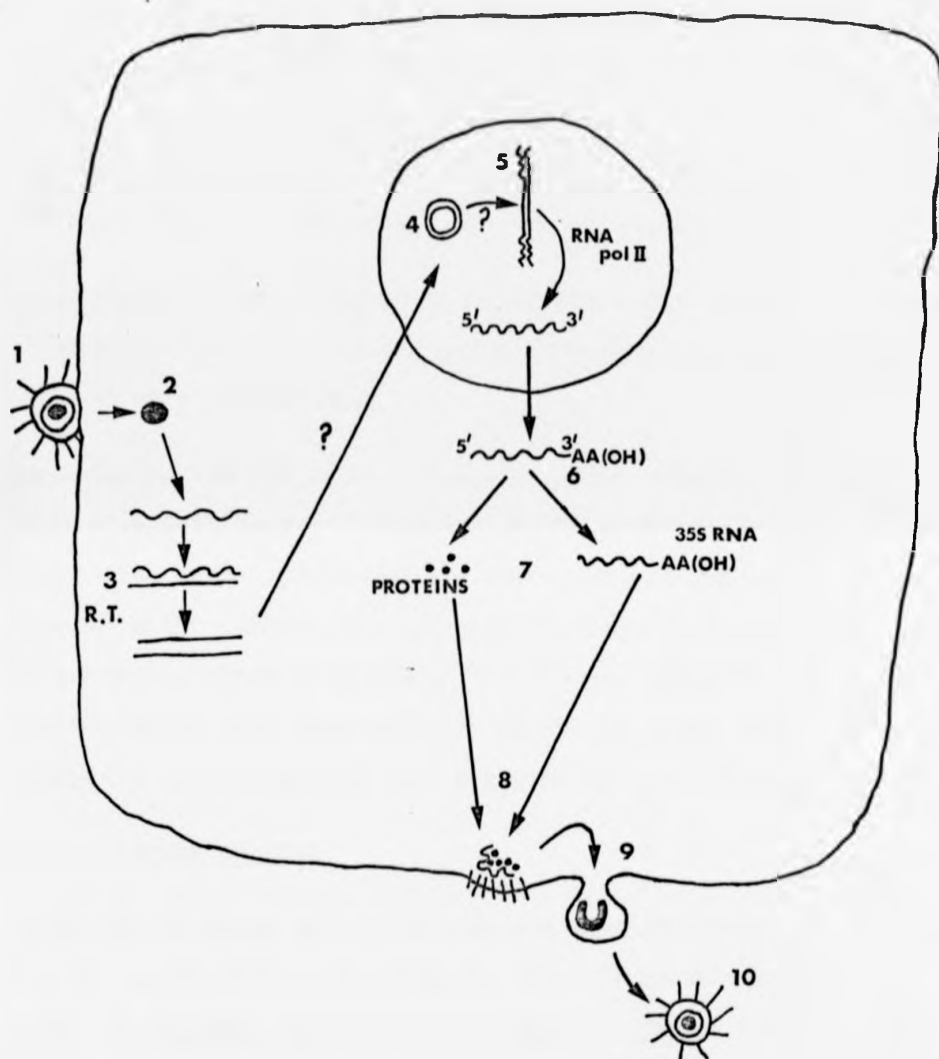


FIGURE 9. Life Cycle of RNA Tumour Viruses

R.T. : Reverse Transriptase

(Adapted from Verma, 1977).

Invertebrate Virology

The aim of this project was to determine if RNA tumour viruses were present within invertebrate cells. The detection of these viruses in insect cells would not only extend the known host range of such viruses but would also indicate the potential of invertebrate cells to act as a reservoir and vector for the RNA tumour viruses.

The existence of the retroviruses in insect cells could also lead to the contamination of preparation of invertebrate viruses disseminated for use as viral pesticides.

We will now review the known viruses of invertebrates and the tissue culture of insect cells. In order to avoid confusion of any viruses detected with known invertebrate viruses, it was important to understand which viruses were already recorded in invertebrate cells. After reviewing these viruses we will discuss the tissue culture techniques which have been employed in the in vitro growth of invertebrate cells enabling such experiments to be performed.

Viruses of Invertebrates

At least 80% of known animal species are invertebrates. A large number of viruses have been shown to exist in invertebrates e.g. Amoebae, Protozoans, Squid, Crab and Oyster. These viruses may be broadly divided into those which are pathogenic to the invertebrate host and those which are transmitted by invertebrate vectors to animal and plant hosts, this latter virus type will be discussed later. The pathogenic viruses of invertebrates are discussed below.

Invertebrate Pathogenic Viruses

Of all the invertebrate viruses by far the most well-studied are the viruses of the insects (Smith, 1976; Tinsley & Harrap, 1978).

The pathogenic viruses of insects may be further divided into viruses which are associated with inclusion bodies and those which are not.

Viruses associated with Inclusion Bodies

Diseases associated with inclusion bodies have been recorded in the Hymenoptera, Lepidoptera and Diptera (Smith, 1967; Vago & Bergoin, 1968). There are four groups of viruses which are associated with inclusion body disease, the Nuclear Polyhedrosis Viruses (NPV), Granulosis Viruses (GV), Cytoplasmic Polyhedrosis Viruses (CPV) and Entomopox Viruses.

The NPV and GV bear similarities in their replication and are classified in the family Baculoviridae (Fenner, 1977).

NPV were first observed in the mid-nineteenth century (Harrap, 1973). The inclusion bodies associated with these viruses are polyhedral in shape and range from 5 nm to 15 nm and occur within greatly enlarged host cell nuclei, e.g. Bombyx mori NPV.

Large numbers of virus particles are occluded into the crystalline protein matrix which comprises the inclusion bodies of NPV.

Baculovirus particles are bacilliform and range in size from 40-70 nm x 250-400 nm and possess a circular dsDNA genome of $58-100 \times 10^6$ daltons molecular weight (Burgess, 1977; Tija et al, 1979).

The virus particles are occluded randomly throughout the protein matrix which preserves virus infectivity under adverse environmental conditions.

The Granulosis Viruses, as stated earlier, are also members of the Baculoviridae. These viruses are also included in a crystalline protein matrix, however the inclusion body is smaller and only single enveloped viruses are included in each capsule shaped polyhedron.

An example of this type of virus is the Choristoneura fumiferana (CV).

A third subgroup of the Baculoviridae are not occluded into crystalline matrices but bear morphological similarities to other baculoviruses e.g. Oryctes virus (Huger, 1966). Larvae infected with NPV or GV become sluggish and may change colour. The integument becomes fragile and infected larvae are often found hanging upside down from plants. Larval death ensues accompanied by epidermal rupture. This rupture releases large numbers of virus inclusion bodies which are then dispersed by wind and rain to contaminate adjacent food plants.

The baculoviruses are only known to multiply in invertebrates and are of quite specific host range. For these reasons, and for those of ecology, they have become used as biological insecticides in preference to chemical agents (Carstens, 1980).

A third group of included viruses are the Cytoplasmic Polyhedrosis viruses (CPV). As their name implies the polyhedra are found in the cytoplasm of the infected cell and they are similar in structure to NPV polyhedra. The viruses contained within the protein matrix are members of the family Reoviridae. Their genome is dsRNA, in ten pieces ranging in M.W. from 0.3×10^6 - 2.7×10^6 daltons total M.W. Virions are of 50-60 nm diameter and possess a RNA-dependent RNA polymerase activity. Virus multiplication occurs in the midgut epithelium of the host and virions are then occluded into the paracrystalline protein inclusion bodies in the cell cytoplasm (Tinsley & Harrap, 1978).

CPV infection causes larvae to cease feeding and reduce in size. Larvae change colour, as described in NPV/GV infection, and pupation may occur, although pupae are often small and adults, if they emerge, are deformed or sterile.

The fourth group of inclusion body viruses are the Entomopox viruses.

These viruses resemble the pox viruses of vertebrates. They are oval or brick shaped and range in size from 230-320 nm in Diptera (Weiser, 1969) to 400 x 250 nm in Coleoptera (Bergoin et al 1971). Their genome is dsDNA of m.w. range 130-240 x 10⁶ daltons. Virus multiplication takes place in leukocytes or adipose cells and inclusion bodies, spherules, may be found in the cell cytoplasm (Devauchelle et al 1971).

Use of Viruses as Pesticides

Over recent years the use of chemical insecticides has come under close scrutiny for biological and ecological reasons.

Firstly the insect pests develop resistance to chemical pesticides very quickly.

Secondly chemical insecticides are non-specific destroying a number of beneficial insects as well as the pests we attempt to control. The chemicals may also persist for unacceptable lengths of time in the environment.

The insect pathogenic viruses, particularly the Baculoviruses, would appear to overcome all these problems. They are naturally occurring insecticides, species specific and relatively cheap to produce. Their paracrystalline protein matrices protect the viruses in the environment and also reduce any possibility of vertebrate infection. However, use of the baculoviruses as insecticides have yielded mixed results. In the U.S.A. the corn earworm (Heliothis zea) has been controlled by viruses whilst in East Africa the armyworm (Spodoptera exempta) has proven more difficult to control by viral methods.

The mixed results may be due to the non-scientific method of dissemination. Instances were recorded of infected larvae simply being mashed in water before being sprayed on crops. By this technique not only baculoviruses may be spread into the environment. It is thus

necessary to establish if any other agent/virus is present within those insect cells used to produce viral insecticides. Viral insecticides, especially in countries of the Third World where large quantities of agricultural crops are destroyed by pests, may become very important in the future. Research is currently being carried out on insecticide production and the effects of baculoviruses on the environment.

The research which was carried out in this thesis was also directed at answering some of these questions.

Viruses associated with Non-inclusion Diseases

There are many invertebrate viruses which are not associated with inclusion bodies and which possess widely differing properties.

The best studied of these 'non-occluded' viruses are the Iridescent viruses, notably the virus of the crane fly Tipula paludosa, first recorded by Xeros (1954). The viruses of this group are so called due to the occurrence of a blue green iridescence within infected host cell tissue which arises as a result of viral multiplication in the host.

Iridescent viruses have been isolated from a large number of invertebrates e.g. mosquitoes (Aedes cantans (Weiser, 1965), A. detritus (Hasan, 1970), midges, (Culicoides sp.) (Chapman et al 1968) stem borer (Chilo suppressalis), (Fukaya and Masu, 1966) and many others (Kelly & Robertson, 1973). A nomenclature for these viruses has been suggested by Kelly and Robertson (1973). There appears to be a degree of serological and genetic homology between some of the viruses, evidence against the theory that each virus isolate is a distinct virus specific to a given host.

Iridescent virus particles are icosahedral and non-enveloped of 130-180 nm diameter. Their genome is double stranded DNA of $130-160 \times 10^6$ daltons m.w. It is known that Type 2 (Sericesthis virus) and Type 6 (Chilo virus) possess an RNA polymerase activity (Kelly and Tinsley, 1973).

Whilst all the iridescent viruses are morphologically similar, and, as stated earlier, there is a degree of serological and genetic similarity between some members of the iridescent group, there is diversity amongst many of their properties (Kelly & Robertson, 1973). Kelly and Robertson (1973) have reviewed their relationship to other morphologically similar viruses isolated from other hosts.

Only over the past decade have non-occluded viruses of smaller size than iridescent viruses been reported. There are now large numbers of such viruses which have been recorded, however few have been intensively studied and characterized.

Perhaps the best studied virus of this group is the Densonucleosis virus (DNV) of the waxmoth Galleria mellonella (Meyanadier et al, 1964). The virus causes a fatal disease in the insect, killing larvae after 4-6 days.

The DNV of Galleria shows several characteristics of the Parvoviruses (Tinsley and Longworth, 1973). The particles are non-enveloped, icosahedral in shape and of 18-26 nm diameter. The particle contains ssDNA of $1.2-1.8 \times 10^6$ daltons m.w. Evidence has been presented to suggest individual DNA molecules from individual particles were complementary (Barwise & Walker, 1970).

A number of small RNA-containing invertebrate viruses have been isolated. Several of these small RNA-containing viruses share biophysical and biochemical properties with the Picornaviruses. The viruses are small, of 20-30 nm diameter containing RNA. The single

stranded RNA genome is of 2.5×10^6 daltons m.w. and virus multiplication takes place in the cytoplasm.

Three Picornaviruses have been isolated from Drosophila spp, P Virus (Plus and Duthoit, 1969) Iota Virus (Jousset, 1970) and C Virus (Jousset et al, 1977). P Virus multiplies in the cytoplasm of Drosophila melanogaster and causes sterility in female flies and a 50% reduction in lifespan. The virus particle is 25 nm in diameter and contains RNA. The particle appears spherical in shape although it may be polyhedral (Teninges and Plus, 1972).

Iota virus was isolated from Drosophila immigrans and is of similar morphology to P Virus. It appears as an electron dense sphere in the electron microscope and confers CO₂ sensitivity on the male host fly.

Isolation of both P virus and Iota virus has been described by Plus et al, (1972).

C virus was also isolated from Drosophila melanogaster and is morphologically similar to P virus. The virus particle is of 30 nm diameter and contains a single-stranded RNA genome (Jousset et al, 1977).

A Rhabdovirus has also been isolated from Drosophila melanogaster, Sigma virus. Virus infection confers CO₂ sensitivity on the host and the virus is transmissible congenitally. Carbon dioxide sensitivity can be demonstrated by exposing flies to an atmosphere rich in carbon dioxide. Flies quickly become anesthetized, however on re-exposure to normal air uninfected flies recover, infected flies remain paralyzed and die after a few hours.

A rhabdovirus-like particle was observed in the tissues of infected flies by Berkalooff et al (1965). Virus particles have also been observed in germinal cells together with virus budding from the cell

surface (Teninges, 1968). Particles observed are bullet-shaped and of 140-180 x 70 nm diameter. The viral genome is single-stranded RNA of $3.5 - 4.6 \times 10^6$ m.w. and the virion contains a RNA-dependent RNA polymerase activity.

Two other virus particles have been isolated from Drosophila sp. A Reovirus-like particle has been isolated from a cell-line of Drosophila melanogaster (Haars et al, 1980). The virus particles contain 10 segments of ds RNA and are of 56 nm diameter.

A virus known as Drosophila X virus has been isolated from Drosophila in vivo and from cultured cell lines, (Teninges, 1979; Teninges et al, 1979). The virus is icosahedral and of 60-70 nm diameter. The viral genome is believed to be dsRNA and has two components of 5S and 14S. It appears that DXV is similar to Infectious Pancreatic Necrosis Virus of the Trout, a reo-like virus (Cohen et al, 1973).

A number of virus-like particles have been observed in both Drosophila flies and derived cell lines (Akai et al, 1967; Filshire et al, 1967; Rae and Green, 1967; Philpott et al, 1969; Gartner, 1971; Miquel et al, 1972; Williamson & Kernaghan, 1972; Plus, 1978). Particles range in size from 40-60 nm and are often of toroidal morphology (see Results Section later).

Drosophila sp. are not the only cells which harbour such small isometric viruses. 6 viruses known to contain RNA have been isolated from honey bees, eg. Acute Bee Paralysis Virus (ABPV) (Bailey et al, 1973). Gibbs et al (1970) have described three small viruses in the termite, and many other small viruses have been observed in the mosquito (Scherer and Hurlbut, 1967), cricket (Meyandier, 1966) and grasshopper (Jutila et al, 1970). We can be sure that as more invertebrates are examined more viruses will join this list.

INVERTEBRATES AS VIRAL VECTORS

Viruses may be transmitted from invertebrates to vertebrates by two methods:

(a) Mechanical transmission. Virus may be acquired by the insect on probing infected plant or animal tissues. Virus is then transmitted to other healthy individuals within the population by further biting and probing.

This mode of transmission does not involve any infection of the vector by the virus. An example of a vertebrate virus being transmitted mechanically by an invertebrate vector is myxamatosis. Both mosquitoes and rabbit fleas transmit the disease and high titres of virus may be found in the skin of the infected host.

(b) Biological Association. Most of the viruses which are carried by invertebrates to infect higher animals also infect and replicate within the vector. Such viruses are called arthropod-borne or arboviruses. This virus replication usually causes no pathological effect in the insect vector.

Main arbovirus vectors are mosquitoes, midges and sandflies (Dipterans) and the ticks and blood-sucking mites (Acarina).

Virus is acquired from an infected animal during a blood-meal to be carried to the next host within the insect saliva. The interval between the acquisition of virus and onward transmission is known as the 'extrinsic incubation period'. During this period the virus multiplies within the arthropod.

The 'extrinsic incubation period' is dependent upon temperature in two ways:

(a) If the arthropod vector is exposed to any extremes of temperature it may die before viral transmission can take place, and

(b) the virus is exposed to a very different host cell temperature within the insect. Virus must multiply within the tissues of the vector at a temperature of approximately 28°C compared to the higher animals' body temperature of 37°C.

This variation in temperature may exert a selection pressure on the virus population within the vector. For example viral enzymes must function at both temperatures sufficiently well to allow virus multiplication. Any adverse effects of temperature difference on enzymatic functions could result in an attenuation of the virulence of the virus.

The largest taxonomic subset of the Arboviruses is the virus family Bunyaviridae. These viruses are spherical, enveloped particles of 90-100 nm diameter. The viral genome is segmented, (3 species), single-stranded RNA of $4-6 \times 10^6$ daltons molecular weight. Virus replication is cytoplasmic.

Rehacek et al (1976) have reported that vertebrate oncogenic viruses may also be transmitted by hematophagous arthropods although this transmission was by mechanical means. No report has been made of a vertebrate oncogenic virus being transmitted by arthropods in the manner of arboviruses.

INVERTEBRATE TISSUE CULTURE

The many advances which have been made in animal virology over recent years owe much to the development of tissue culture techniques and the ability to grow animal cells in vitro.

There are many advantages which arise from the ability to grow cells in vitro:

- (i) control over growth conditions. Physical factors may be closely controlled e.g. temperature, atmosphere etc. Chemical factors may also be closely controlled by choice of culture medium (see later);
- (ii) growth of large quantities of cell tissue in small amount of space;
- (iii) ability to maintain cells free of other biological contamination;
- (iv) cell cultures may be stored under liquid nitrogen to maintain original cell stock;
- (v) viruses may be grown to very high titres by the use of tissue cultured cells.

The first insect cell line was established in tissue culture in 1962 by Grace. Since then a large number of insect cell lines have been established many derived from moths, mosquitoes, leafhoppers and fruit fly.

The major difficulty in insect tissue culture is the small size of the animal from which cells are to be taken.

Most insect tissue cultures were established by crude trypsinization of whole embryos or larvae. Cell lines which were so derived were therefore composed of unspecific cell types. Cell lines continue to be established in this manner since it is extremely difficult to obtain

sufficient quantities of specific tissues from most species. This is not only due to the small size of the insect but also to the physical problem of growing sufficient numbers of the insect owing to insect life cycle and/or breeding habits.

The two most important factors affecting the growth and morphology of insect cell cultures are the cell growth medium and the temperature of the culture.

In recent years the constituents of the culture medium for invertebrate cells have undergone revision. Until recently it was believed that insect hemolymph was essential for sustained cell growth. This belief was contradicted by the development of hemolymph free media by Mitsuhashi (1967) and Kitamura (1966).

Hemolymph was found to be unnecessary if foetal calf serum was added to 10-20%, indeed, most insect cell lines are now grown in media containing vertebrate serum.

As in vertebrate cell culture serum appears necessary for insect cell attachment to substrate and for subsequent cell division.

Cell morphology is affected by temperature. The normal temperature range of insect cells is 24-30°C. However at higher or lower temperatures the cell morphology changes. At lower temperatures cells become round however they remain attached to the substrate. At higher temperatures cell detach more easily from the substrate, eventually leaving the substrate whilst retaining their flattened shape.

At 37°C cells begin to take on a rough, pitted appearance under the phase contrast microscope and after 48 hours begin to degenerate. Cells seldom survive at this temperature for over 7 days.

Invertebrate cell lines may be stored under liquid nitrogen, no ill-

effects being observed upon subsequent culture of cells subjected to this procedure.

Temperature range for cell growth is the main difference between vertebrate and invertebrate cells. Vertebrate cells grown within temperatures of 32-40°C while invertebrates grow at temperatures between 16°C and 32°C. This point is important and will be discussed later in the Results Section.

MATERIALS

1. Chemicals

5-Bromo-2' deoxyuridine (BDUR); Caffeine; Clelands Reagent (dithiothreitol) (DTT); DEAE-Dextran; Deoxyadenosine 5'-triphosphate, Disodium Salt Equine Muscle (dATP); Deoxyguanosine 5'-triphosphate, Type II S Disodium Salt Equine Muscle (dGTP); Deoxythymidine 5'-triphosphate, Sodium Salt (dTTP); 5-Iodo-2' deoxyuridine (IDUR); 2-mercaptoethanol, type I; Polyadenylic acid (poly (rA)); Polybrene (Dimethyl-1,5-diazaundecamethylene polymethobromide); Trizma base (tris (hydroxymethyl) aminomethane), were purchased from Sigma Chemical Co., London.

Oligo(dT)-cellulose, type 3 and Poly methyl cytidylate oligodeoxyquanylate (poly(rCm).oligo(dG)), were obtained from Collaborative Research Inc, Waltham, Mass., U S A.

Oligo deoxythymidylate, (Oligo(dT)₁₂₋₁₈); Poly deoxyadenylic acid. oligo-deoxythymidylate, (poly(dA).oligo(dT)₁₂₋₁₈) and Poly cytidilate oligodeoxyquanylate, (poly(rC).oligo(dG)₁₂₋₁₈) were purchased from P-L Biochemicals Inc., Milwaukee, Wis., U S A.

Sodium lauryl sulphate, (SDS), especially pure grade, Triton X-100 (iso-octylphenoxy-polyethoxyethanol), and Folin and Ciocalteu Reagent were supplied by B D H Chemicals, Poole, Dorset.

All other chemicals were the best grade available.

2. Radio-labelled Compounds

All radiolabelled materials were purchased from the Radiochemical Centre, Amersham, Bucks. Specific activities were as follows:

Deoxycytidine 5'-[α - ^{32}P] triphosphate, (^{32}P -dCTP), 400 Ci/mmol

[8- ^3H] Guanosine 5' triphosphate, (^3H -dGTP), 10-20 Ci/mmol

Phosphorous-32, (^{32}P), 10mCi/ml

Poly [8- ^3H] adenylic acid, (^3H -poly(rA)), > 15 Ci/mmol, 1.98 Ci/ μg

[methyl- ^3H] Thymidine 5' triphosphate, (^3H -dTTP), 40-60 Ci/mmol

[5- ^3H] Uridine, 25-30 Ci/mmol

3. Biological Materials

Pancreatic ribonuclease A, (RNaseA), Type IA from Bovine Pancreas, ribonuclease T₁, Type III from Asperigillus oryzae (RNase T₁) and Bovine Albumin Fraction V were purchased from Sigma Chemical Co., London.

Deoxyribonuclease, ribonuclease-free, electrophoretically purified, (DNase) was supplied by Worthington Biochemical Corp., New Jersey, U S A.

Foetal Bovine and Newborn Calf Serum were provided by Flow Laboratories Inc., Irvine, Scotland.

Crystamycin (containing 600 mg (1 million units) of Penicillin to 1 g of streptomycin) was purchased from Glaxo Laboratories Ltd., Greenford, Essex.

Bactotryptone medium; Bactotryptose broth; Lactalbumin hydrolysate and Yeastolate were purchased from Difco Laboratories, East Mollesley, Surrey.

Avian Myeloblastosis Virus Reverse Transcriptase was a gift from Dr J W Beard, Life Sciences Inc., St Petersburg, Florida, U S A , and was used at 12×10^3 units/ml.

Goat Anti-AMV Reverse Transcriptase was a gift from Dr R E Wilsnack, Huntingdon Research Centre, Brooklandville, Maryland, U S A.

Rous Sarcoma Virus B77 Clone V was a gift from Dr J Wyke, ICRF, Mill Hill, London.

4. Tissue Culture Media

Eagle's DMEM was prepared from a concentrate from Flow Laboratories and was supplemented with 10% (v/v) newborn calf serum, 100 units/ml of crystamycin and was adjusted to pH 7.2 with 5% (w/v) sodium bicarbonate solution.

Leibovitz Medium (L-15) x 1 and Schneiders Drosophila Medium (Revised) x 1 were purchased from Gibco Bio Cult Ltd., Glasgow, Scotland. L-15 was supplemented with 10% (v/v) heat inactivated foetal calf serum, Schneiders with 10% (v/v) foetal calf serum.

Calf Serum for use in culture medium was inactivated by heating in a water bath to 56° for 30 minutes, (Δ FCS or Δ CS).

Mitsubishi & Marmarosch Medium x 1 (MM) was purchased from Flow Laboratories, Irvine, Scotland and was supplemented with 10% (v/v) foetal calf serum.

BML/TC10 medium was prepared by the method of Gardiner and Stockdale (1975), and was supplemented with 10% heat inactivated foetal calf serum.

Vitamins (x 100) for Basal Eagle Medium were purchased from Flow Laboratories, Irvine, Scotland.

MK, a modification of Kitamuras solution, was made up according to the method of Varma & Pudney (1969) and consisted of NaCl 650 mg; KCl 50 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 10 mg; KH_2PO_4 10 mg; D-glucose 400 mg; lactalbumin hydrolysate 650 mg; yeastolate 500 mg; distilled water 80 ml. pH was adjusted to 7.0 with 2% KOH and the medium passed through 0.22 micron Millipore filters (Millipore (UK) Ltd., London).

VP12 medium was devised by Varma & Pudney (1969) and was made up of NaCl 390 mg; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 55 mg; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 110 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 120 mg; KCl 55 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 40 mg; D-glucose 200 mg; choline chloride 25 mg; inositol 40 mg; NaHCO_3 50 mg; lactalbumin hydrolysate 500 mg; bovine albumin 100 mg; 5% glutamine 0.6 ml; Basal Eagle medium Vitamins x 100 2 ml; distilled water 97.4 ml. pH was adjusted to 7.0 with 2% KOH and the medium filtered through 0.22 micron Millipore filters.

The media above were all tested for sterility by inoculation into Nutrient Broth (13 g/l) and incubation at 37°C for 5 days.

5. Buffers and Solutions

All buffers and solutions except Basic Minimal Salts Solution (BMSS), 'Trypsin' and Sucrose were sterilized by autoclaving at 15 psi for 20 minutes. Sucrose solutions were sterilized by autoclaving at 10 psi for 10 minutes. BMSS and Trypsin were sterilized by filtration through 0.22 μ m Millipore filters.

Basic Minimal Salts Solution was 3.8 mM- $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; 2.1 mM- NaHCO_3 ; 70 mM KCl; 4.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 5.6 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; 5.6 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; pH 6.2.

TNE was 50 mM tris containing 100 mM NaCl and 1 mM ethylene diamine tetra-acetic acid (disodium salt(EDTA)), pH 7.4. TNE was made up 5 fold concentrated and diluted with sterile distilled water before use.

TLES was 50 mM-tris, pH 7.6; 100 mM LiCl; 1 mM EDTA; 0.1% (v/v) SDS.

STE was 1 mM EDTA; 100 mM NaCl; 10 mM tris, pH 7.5.

PBS was 0.14 M NaCl; 2.7 mM KCl; 8.1 mM Na_2HPO_4 ; 1.5 mM KH_2PO_4 ; pH 7.4.

DNA buffer was 20 mM Tris.HCl; 200 mM NaCl; 10 mM EDTA, pH 7.5. The buffer was made up 10 fold concentrated and diluted with sterile distilled water before use.

DNA suspension buffer was 10 mM Tris and 0.5 mM EDTA, pH 7.5.

Sucrose solutions were made up in the appropriate buffer and sterilized by autoclaving at 10 psi for 10 minutes. Sucrose solutions were made up w/w except where stated.

'Trypsin' for cell passage was 8 g NaCl, 0.38 g KCl, 0.1 g Na_2HPO_4 ,

1 g D-glucose, 3 g tris, 15 ml 1% (w/v) phenol red, 10^5 units of penicillin, 0.1 g streptomycin and 2.5 g of trypsin in 1 litre of distilled water (pH 7.7).

'Versene' for cell passage was 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 , 0.2 g KH_2PO_4 , 0.2 g EDTA, 1% (w/v) phenol red in 1 litre of distilled water.

Binding buffer, for use with Oligo(dT).cellulose columns, was 50 mM tris, pH 7.4; 0.3 M LiCl; 1 mM EDTA; 0.1% SDS.

Elution buffer for Oligo dT-cellulose columns was 10 mM-tris, pH 7.4; 0.1% SDS.

Veronal Acetate Buffer, used in Kellenburger buffer, was 2.94 g sodium veronal, 1.17 g anhydrous sodium acetate and 3.4 g NaCl in 100 mls distilled water.

Kellenburger buffer was 5 mls veronal acetate buffer, 13 mls distilled water, 7 mls of 0.1N HCl and 0.25 mls of 1M CaCl_2 . pH was adjusted to pH 6 with HCl.

6. Scintillation Mixtures

Toluene scintillation mixture was 8 g of PPO in 2 litres of toluene.

Triton-toluene scintillation mixture was 1 volume of Triton X-100 and 2 volumes of toluene scintillation mixture containing 0.266 g/l final concentration of POPOP.

Instagel was purchased from Packard Instrument Company Inc., Downers Grove, Illinois, USA.

METHODS

Cell Cultures

The cell lines and media used in this work are listed in Table 3.

The cell lines Aedes aegypti, Anopheles stephensi, Anopheles gambiae, Triatoma infestans, Rhipicephalus appendiculatus²⁴³, Rhipicephalus appendiculatus²⁵⁷, and Aedes pseudoscutellaris were the gift of Dr M Pudney, London School of Hygiene and Tropical Medicine. The remaining invertebrate lines were obtained from the NERC Unit of Invertebrate Virology, 5 South Parks Road, Oxford, which obtained the Drosophila melanogaster cell line as a gift from Dr P Scotti, Entomology Division, DSIR, Auckland, New Zealand.

Invertebrate cells were grown in 25 cm, 75 cm and 150 cm plastic flasks unless stated otherwise.

To prepare cells for seeding, the flask containing cells and culture fluid was shaken until the cells detached from the flask. Cells were dispersed by pipetting and passed 1 : 10 into fresh medium. Flasks were incubated at 28°C until the monolayer reached confluence.

Vertebrate cells were grown in plastic flasks and 2.5 l glass roller bottles, and were passed as follows. The cell culture fluid was poured off and the monolayer washed with versene/trypsin, (4:1 v/v) eg 10 ml per roller bottle. Cells were then incubated at 37°C in versene/trypsin until the cells had detached from the substrate. Cells were dispersed by pipetting and added to an equal volume of fresh medium. The cells were then passed 1 : 10 from flasks or 1 : 4 from roller bottles. Bottles were gassed for 10 seconds with 5% CO₂/95% air

Table 3.

Table of the cell lines and their growth media used in this work.

MM/VP12 was a 1 : 1 mixture (v/v) of MM and VP12. Heat inactivated foetal calf serum was added to 10%.

MK/VP12 was a 1 : 1 mixture (v/v) of MK and VP12. Heat inactivated foetal calf serum was added to 10%.

TABLE 3.

Cell Line	Common Name	Medium	References
<u>Drosophila melanogaster</u>	Fruit Fly	Schneiders	
<u>Aedes aegypti</u>	Mosquito	MM/VP12 [*]	Varma & Pudney 1969
<u>Anopheles stephensi</u>	Mosquito	MK/VP12 [‡]	Pudney & Varma 1971
<u>Anophelesambiae</u>	Mosquito	MK/VP12	Marhoul & Pudney 1971
<u>Triatoma infestans</u>	Bug	L15	Pudney & Lanar 1977
<u>Rhipicephalus appendiculatus</u> ²⁴³	Tick	L15	Varma <u>et al</u> 1975
<u>Rhipicephalus appendiculatus</u> ²⁵⁷	Tick	L15	Varma <u>et al</u> 1975
<u>Aedes pseudoscutellaris</u>	Mosquito	MM/VP12	Varma <u>et al</u> 1974
<u>Spedoptera frugiperda</u>	Armyworm	BML/TC10	
<u>Spedoptera littoralis</u>	Armyworm	BML/TC10	
<u>Trichoplusia ni</u>	Cabbage Looper	BML/TC10	
<u>Heliothis zea</u>	Cotton bollworm	BML/TC10	
<u>Predenia littura</u>	Armyworm	BML/TC10	
<u>Choristoneura fumiferana</u>	Spruce budworm	BML/TC10	
<u>Malacasoma disstriae</u>	Tent Caterpillar	BML/TC10	
<u>Carpocapsa pomonella</u>	Codling Moth	BML/TC10	
<u>Aedes albopictus</u> ^{WAR}	Mosquito	L15	
<u>Aedes albopictus</u> ^{MM}	Mosquito	MM	
NIH 3T3	Mouse Embryo Fibroblasts	DMEM	
NRK	Normal Rat Kidney	DMEM	
XC (NRK $\xrightarrow{\text{RSV}}$ XC) <u>in vitro</u>	Rat Tumour Cell Induced by Prague Strain of RSV	DMEM	
CAT	Canine Thymus	DMEM	
RK1X	NRK infected with XLV (KMSV)	DMEM	
1855.B4	NRK transformed by HaMSV	DMEM	

before incubation at 37°C. Flasks were incubated in an humidified incubator at 37°C in an atmosphere of 5% CO₂/95% air.

Cell cultures were incubated until the monolayers were confluent.

Chick embryo fibroblasts (CEF) were prepared by the method of Morser et al (1973).

Virus

The Kirsten murine sarcoma virus (KMSV) used was originally obtained from Dr S Aaronson, National Institute of Health, Bethesda, Maryland, U S A. The virus was propagated in NRK cells, medium from these cells being harvested every 4 hours for 2 days. The supernatant fluid was clarified by a low speed centrifugation and frozen at -70°C. Further purification of the virus prior to virus 'core' preparation involved pelleting the virus by centrifugation at 47,000 g for 3 hours. The virus pellet was then cushioned on to 40% (w/v) sucrose/STE through 15% (w/v) sucrose/STE by spinning at 60,000 g for 2 hours. Virus was then placed on 25-50% (w/v) sucrose/STE density gradients, and centrifuged at 50,000 g for 12 hours. Fractions with a density of 1.16 g/ml were pooled and frozen at -70°C until required.

XLV(KMSV) was a 'pseudotype' virus prepared by infection of cells transformed by KSV with xenotropic leukemia virus and therefore consists of a KMSV genome surrounded by a xenotropic virus coat. This virus was obtained from the National Cancer Institute, Bethesda, Maryland, U S A , and was propagated in NRK cells. Medium from these cells was harvested every 6 hours for 3 days, and fluids were clarified by low speed centrifugation and frozen at -70°C.

MSV Focus Assay

Hartley and Rowe (1966) developed a quantitative in vitro assay for MSV and a similar procedure was followed in this work.

60 mm plastic Petri dishes were seeded at 3×10^5 cells/plate with NRK cells to achieve ~50% confluency.

Cells were incubated for 24 hours at 37°C in a humidified, 5%CO₂/95% air, atmosphere.

The monolayer was observed to ensure an even distribution of cells with no clumps of cells which could lead to pseudofoci. Fluid was removed with a Pasteur pipette by suction and the monolayer treated with 2 mls of DEAE-dextran at 25 µg/ml, (Duc-Nguyen, 1968). Plates were incubated at 37°C for 30 minutes.

Dextran was removed and plates were inoculated with dilutions of virus, disrupted cells or medium at 0.4 ml per plate. Cells were disrupted by 4 cycles of freeze/thawing. Plates were incubated at 37°C for 30 minutes with occasional rocking to distribute inoculum evenly. After incubation, 4 ml of medium was added. Assays were performed in duplicate.

Plates were incubated at 37°C in a humidified, 5% CO₂/95% air, atmosphere and the culture fluid changed on the 2nd or 3rd and 5th days.

Foci were counted when the monolayer was confluent (usually between 5 and 7 days from inoculation) on a dissecting microscope. Dishes were drained completely before reading; the dishes remaining sterile for return to the incubator if foci had not developed.

MuLV Plaque Assay

The plaque assay technique was a modification of that described by Klement et al., (1969) and Rowe et al., (1970).

60 mm plastic Petri dishes were seeded at 30×10^5 cells/plate with NIH 3T3 cells in DMEM + 10% FCS. Plates were incubated at 37°C in a humidified, 5% CO_2 /95% air, atmosphere for 24 hours.

The quality of the monolayer was checked by microscopic examination and the culture fluid removed by suction. The cells were then treated with DEAE-dextran as above.

After 30 minutes the dextran was removed and the monolayer washed once with medium. Virus samples or control fluids were added 0.4 ml/plate using 2 dishes for each dilution. 4 mls of DMEM + 10% FCS per dish were then added and the plates returned to the incubator. Culture medium was changed on the 2nd and 4th days after infection.

On the 6th day after infection, culture fluid was removed from the plate and the cell sheet irradiated for 20 seconds with ultra-violet radiation. 10^6 XC cells were then added per dish in 4 mls of DMEM + 10% FCS. Culture fluid was then changed, carefully as the cells sheet was fragile after irradiation, on the first and second day after irradiation.

On the 4th day after irradiation, the cells were washed x 1 with saline and fixed with methanol, 2 ml/dish, for 15 minutes.

The methanol was removed and the cell sheet allowed to air dry for 2 hours and then stained with 0.11% Basic Fuschin, 0.33% Methylene Blue in methanol for 1 hour.

After staining, the stain was removed and the cell sheet washed with tap water. Plaques could then be counted.

Reverse Transcriptase Assay

The assay was a modification of that used by Baltimore (1970) and Temin and Mizutani (1970). Assays were performed using 10 μ l of sample, made 0.1% with Triton X-100 and added to 50 μ l of a reaction mixture containing 62.5 mM-Tris, 75 mM-KCl, pH 7.8; 0.625 mM-MnCl₂; 2.5 mM DTT; 1 μ g/ml poly(rA); 0.18 units/ml oligo(dT)₁₂₋₁₈ and 6.25 Ci ³H-dTTP.

When poly(rA).oligo(dT)₁₂₋₁₈ and poly(rC).oligo(dT)₁₂₋₁₈ were used in the assay, 0.1 units of each were added. When the specific template primer poly(rCm).oligo(dG) was used, 0.2 units were added to each reaction mixture. When poly(rC) or poly(rCm) were the added templates, ³H-dGTP replaced the ³H-dTTP as label.

Mixtures were incubated at 37°C for 1 hour. At the end of this period 50 μ l samples were spotted on Whatman DE 81 filter paper, washed five times in 2% disodium hydrogen orthophosphate, once in distilled water, twice in ethanol and twice in diethyl ether. These papers were then dried under an infra-red lamp, Triton-toluene scintillant was added and the samples counted. The procedure described above was also used to determine the effect of varying the concentration and type of divalent cation supplied to the enzyme while utilizing poly(rA).oligo(dT) as template primer.

Endogenous Reverse Transcriptase Reaction

Assays were again performed using 10 μ l of sample made 0.1% with Triton X-100. Samples were added to 40 μ l of a reaction mixture of final concentrations 50 mM-tris; 60 mM-KCl, pH 7.8; 6 mM MnCl₂; 2 mM-dithiothreitol; 0.5 mM dATP, dTTP and dGTP and 2 Ci of ³²P-dCTP,

Calf thymus DNA fragments ($6\mu\text{g}$), (prepared by Dr D Kohne, Torrey Pines Laboratories, La Jolla, California, U S A), were also added to the reaction mixture to act as primers (Taylor et al, 1976).

Mixtures were incubated at 37°C for 1 hour. Samples were then treated as previously described for the reverse transcriptase assay. In control experiments, samples were pre-incubated at 37°C for 30 minutes with RNase (pancreatic RNase A, 1 mg/ml; RNase T_1 , 10^3 units/ml, boiled before use for 2 minutes), and then assayed for their endogenous polymerase activity as described above.

RNase H Assay

Retroviruses possess the enzyme activity RNase H (Moelling et al, 1971; Moelling, 1974). To assay for this enzyme an RNA.DNA template primer was prepared.

$17.5\mu\text{l}$, ($0.44\mu\text{g}$) of ^3H -poly(rA) was made up to $70\mu\text{l}$ with sterile distilled water and was added to $70\mu\text{l}$ of oligo(dT) ($250\mu\text{g/ml}$). This mixture was added to $140\mu\text{l}$ of 0.1 M Tris.HCl, pH 7.9; 20 mM MgCl_2 ; 0.2 M NaCl and incubated at 37°C for 15 minutes, followed by 15 minutes at room temperature.

Reaction products were then tested with RNase, as above, and TCA precipitated. $10\mu\text{l}$ of sample was taken and added to $40\mu\text{l}$ of 0.1M Tris.HCl, pH 7.9; 20 mM MgCl_2 ; 0.2 M NaCl. 0.9 ml of 0.4 M NaCl was added to the sample and $100\mu\text{l}$ of 100% TCA and the mixture put on ice for 5 minutes. Samples were then passed over Millipore HA filters of $0.45\mu\text{m}$ pore size and the fluid collected in a glass scintillation vial. Filters were washed with 2 ml of 6% TCA and dried under an infra-red lamp. Instagel was added to the filtration fluids

and samples counted. Filters were counted in triton X-100 scintillant.

Results of this synthesis, shown in Table 4, indicate that a poly(rA). oligo(dT) RNase resistant hybrid was synthesized with the 3H label in the poly(rA) molecule.

Electron Microscopy

Cells were prepared for electron microscopy by one of the methods below:

- a) Cells were fixed in 2% glutaraldehyde and pre-stained with 1% osmium tetroxide. After dehydration through a series of graded alcohols, the cells were placed in acetone and embedded in Epon 812.
- b) Cells were suspended in Kellenburger buffer, (Ryter & Kellenburger, 1958), pelleted and fixed with osmium tetroxide and Kellenburger fixative, (1 g bactotryptone medium and 0.5 g NaCl in 100 mls sterile distilled water), 10 : 1, overnight. Cells were diluted with Kellenburger buffer, pelleted and resuspended with a small amount of buffer. Cells were then embedded in 1.5% Agar. The cells in agar were cut into 2 mm cubes and stained with uranyl-acetate for 2 hours. Stained cells were washed twice with Kellenburger buffer and dehydrated via a series of graded alcohols. Cells were then placed in propylene oxide for 20 minutes followed by 1 hour in a 50 : 50 propylene oxide Araldite mixture with gentle shaking. Next day cells were embedded in Araldite.

After sectioning on an LKB microtome, the cells were stained with 2% uranyl acetate and 1% lead citrate. Sections were then observed in either an AE16B or JEOL Temscan electron microscope at 60 KV accelerating voltage.

TABLE 4

SAMPLE	% TOTAL COUNTS PRECIPITABLE	
	^3H Poly(rA)	^3H Poly(rA).oligo(dT)
UNTREATED	88.1	92.5
+ RNase	7.34	89.5

Table showing percentage of ^3H -labelled counts precipitable as poly(rA) and poly(rA).oligo(dT) molecules when untreated and treated with RNase.

Preparation of Virus Cores

Purified KMSV (0.5 mg) was treated with 1% Triton X-100 for 10 minutes at 0°C prior to banding on 15 to 64% sucrose/STE density gradients at 110 000 g for 16 hours. The 1.20 to 1.22 g/ml gradient fractions were pooled and utilized in template primer experiments.

Preparation of 1.22 g/ml Fraction from D.melanogaster Cells

D.melanogaster cells were washed free of medium and disrupted by four cycles of freezing and thawing. A sample of disrupted cells was layered on to a 20 to 50% sucrose/PBS gradient and centrifuged at 110 000 g for 16 hours. The gradient was fractionated using a "Densi-flow" (Buchler Instruments, Baird and Tatlock, London) and pumping off from the top. Fractions were assayed for reverse transcriptase activity as above.

The fractions with maximum enzyme activity were pooled and used in later experiments. Maximum activity was usually found at 1.22 g/ml, although densities as low as 1.20 g/ml were recorded in some experiments. A similar procedure was followed in the assay of several other insect cell lines.

Sucrose Gradient Centrifugation of Disrupted Insect Cells

As for preparation of 1.22 g/ml fraction of Drosophila melanogaster cells.

Preparation of RNA from 1.22 g/ml Fraction of D.melanogaster Cells

D.melanogaster cells were grown on 12 cm glass Petri dishes on 150 cm² plastic flasks. When the cells were 50% confluent, (approx 20×10^6 cells/dish), the medium was replaced with BMSS and the cells were incubated for a further 12 hours. After this time 1mCi of ³H-uridine was added. The 1.22 g/ml fraction was then prepared from these cells as described above.

0.2 g of oligo(dT)-cellulose was suspended in elution buffer at 30°C. The suspension was poured into a water jacketed column, washed at 4°C with elution buffer and then binding buffer. Peak fractions of polymerase activity were made 1% with β -mercaptoethanol and 1% with SDS and passed through the oligo(dT) column. The column was washed with 15 ml of binding buffer. The temperature of the column was then raised to 30°C and elution buffer was added. Fractions of 0.5 ml were collected and the radioactive fractions were pooled for analysis on sucrose gradients.

Sucrose Gradient Analysis of RNA

For analysis of high molecular weight RNA, samples were analysed on 17-35% sucrose gradients in TLES. Gradients were centrifuged for 1 hour and 50 minutes at 100 000 g. Fractions were collected and assayed for radioactivity or O. D. To determine the effect of denaturation on the RNA, samples were heated to 100°C for 1 minute and cooled rapidly on ice. Samples were then loaded on to 15 to 30% sucrose/TLES gradients and centrifuged at 140 000 g for 13 hours. In both types of gradient appropriately sized RNA markers were included.

Induction of Insect Cells

Insect cells were grown in 60 mm plastic Petri dishes until they reached 50% confluency. Culture medium was removed and varying concentrations of IDUR, BDUR and Caffeine in 4 ml of culture medium were added. Cells were incubated at 28°C overnight in the dark. Culture medium was removed, 4 ml/dish of fresh culture medium added, and the cells subsequently incubated at 28°C in the dark over a period of 7 days.

Various parameters were determined, protein concentration, cell numbers and poly(rA).oligo(dT)₁₂₋₁₈ utilization (see above).

Protein concentration was determined by the method of Lowry et al., (1951). Method is outlined in the legend to Figure 10.

Cytotoxicity of the inducing agents was determined as above except poly(rA).oligo(dT)₁₂₋₁₈ utilization was not determined.

Induction of D.melanogaster cells on a larger scale was performed in 150 cm³ tissue culture flasks, using 10 ml of IDUR at a concentration of 25 µg/ml. After 24 hours, the medium was removed and replaced with 20 ml of fresh medium. Cells were incubated at 28°C for 4 days in the dark.

After incubation, the induced fluids were harvested every 4 hours for 2 days. After clarification of the fluids by a low speed centrifugation, the Drosophila 'particle' was pelleted by centrifugation at 47 000 g for 3 hours. Pellets were resuspended in a small volume, 200 µl, of 50 mM-Tris, pH 7.5 and 2 mM NaCl. This suspension was placed on 25-50% sucrose/TNE gradients and centrifuged at 50 000 g for 12 hours. Fractions were collected and assayed for their rA.dT utilization as above.

Preparation of Labelled RNA from Induced Fluids

40 flasks were labelled with 0.5 mCi ³²P post induction in 20 ml of fresh medium. Fluids were then harvested as above.

³²P labelled RNA was prepared from fractions with maximal enzyme activity as follows.

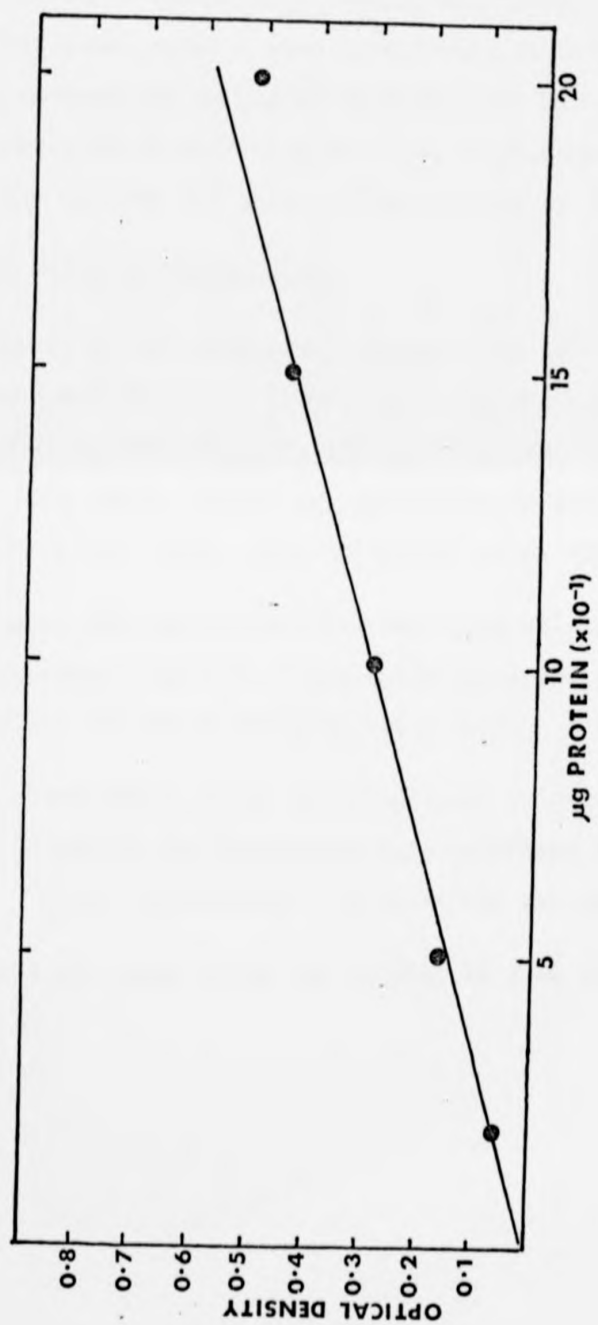
Pellets were resuspended in STE by washing gently. Once homogeneity was achieved, 100 µl of β mercaptoethanol and 200 µl of 20% SDS were added per 10 ml of suspension. After swirling for 20 seconds, lysis was achieved and an equal volume of pre-cooled, (4°C), phenol/chloroform (50 : 50, v/v) was added. This mixture was shaken for 10 minutes and then centrifuged at low speed, (3000 g) for 15 minutes to separate the phases. The aqueous phase was removed and further

Figure 10. Protein Calibration Curve obtained by the method of
Lowry et al (1951)

Known quantities of BSA were assayed in the method described below.

0.5 ml of 1% CuSO_4 and 0.5 ml of 2% sodium potassium tartrate were added to 50 mls of 0.1N NaOH, 2% Na_2CO_3 . To each 0.5 ml of sample, 2.5 mls of this solution was added. Samples were mixed well and allowed to stand at room temperature for at least 10 minutes.

0.25 ml of 1N Folin reagent was added to each sample. Samples were mixed and left to stand for 30 minutes. Absorbance was measured at 650 nm and concentration of protein in the sample obtained from the calibration curve obtained using BSA as sample standard.



extracted with phenol/chloroform until the phase interface was clear. The aqueous phase was removed and two volumes of 100% ethanol at -20°C were added. Samples were precipitated overnight at -20°C . After precipitation, samples were centrifuged at 3000 g for 30 min, the ethanol removed and 50 μl of 50 mM-Tris pH 7.5 and 2 mM NaCl added. These samples were then analysed on sucrose gradients as above and were assayed for their susceptibility to RNase and DNase.

Infection of Cells by Retroviruses

Cells were grown to 50% confluency (approx. 3×10^5 cells per plate), medium removed and the cells treated with either polybrene, (4 $\mu\text{g}/\text{ml}$), when using RSV, or DEAE-dextran, (25 $\mu\text{g}/\text{ml}$), when other viruses, KMSV, XLV (KMSV), were used. Virus was inoculated in the following virus : cell ratios; KMSV, 3:1, XLV(KMSV), 1:10, RSV, 1:1.

After 30 minutes dextran was removed and replaced with fresh culture medium. Polybrene remained on the cells until the culture medium was depleted and had to be replaced, (2-3 days).

After dextran treatment, virus was inoculated in 0.4 ml of medium and allowed to adsorb for 30 minutes in a humidified, 5% CO_2 /95% air atmosphere. After incubation, 4 ml of medium was added.

When Polybrene was used, virus was inoculated into the Polybrene culture medium.

Preparation of DNA from Cells

20 mls of 10 mM-Tris, 0.5 mM EDTA, pH 7.5 was added to the cells. An equal volume of isoamyl alcohol : chloroform (1 : 24) was added to the cell suspension and made 1.5% with SDS. This solution was shaken for 5 minutes at 37°C. 4-Amino salicylic acid was then added to 4%. After addition of an equal volume of phenol/chloroform (50 : 50), the mixture was shaken for 5 minutes. After shaking, the mixture was centrifuged for 15 minutes at 3000 g and the aqueous phase removed. The phase interface was re-extracted until all visible traces of protein were removed. Aqueous phases were pooled and two volumes of 100% ice cold ethanol poured onto the surface of the liquid. DNA was spooled from the ethanol/phase interface, washed in 70% ethanol and 100% ethanol and blown dry.

DNA was resuspended in 10 mM-Tris, 0.5 mM EDTA, pH 7.5 overnight at 4°C and scanned in an ultraviolet spectrophotometer. DNA was then treated with RNase and extracted as above.

ISOLATION OF LOW MOLECULAR WEIGHT DNA FROM CELLS

DNA was isolated by the Hirt fractionation technique (Hirt, 1967).

Medium was removed from cells and washed with PBS 3 ml of 0.6% SDS, 0.01 M EDTA, 10 mM Tris pH 7.5, were added to each 150 cm³ flask. Cells were left at room temperature for 15 minutes until completely lysed. Lysate was poured into a 50 ml Oakridge tube and made up to 1M NaCl. The sample was mixed gently by tube inversion 10-15 times and left at 4°C overnight.

Next day sample was centrifuged at 17,000 g for 30 minutes in a pre-chilled rotor. DNA and SDS sedimented, RNA and any free viral DNA, remained in the supernatant.

DNA EXTRACTION

(i) Supernatant

4 amino-salicylate was added to 4% w/v Supernatant DNA was extracted twice with phenol/chloroform (50:50) and precipitated under ethanol overnight at -20°C.

(ii) Pellet

Pellet was redissolved overnight in the smallest possible volume of 10 mM Tris, 0.5 mM EDTA, pH 7.5.

(i) Supernatant

Next day DNA was pelleted as above and resuspended in 10 mM Tris 0.5 mM EDTA, pH 7.5. Sample was made to 1M LiCl and precipitated on ice for 2 hours. DNA was pelleted by spinning at 3000g for 15 minutes. Supernatant was diluted to 0.5 M salt and precipitated under 2 volumes of ethanol overnight at -20°C.

(ii) Pellet

4 amino-salicylate was added to 4% and made up to 150 mM NaCl. Sample was extracted twice with phenol/chloroform (50:50) and spooled under ethanol.

INDUCTION OF INSECT CELLS : CYTOTOXICITY TESTING

Iododeoxyuridine, Bromodeoxyuridine, and Caffeine were known to have a cytotoxic effect on cell cultures. A series of experiments were carried out on three cell lines which had shown significant levels of rAdT utilizing activity to determine the cytotoxic effect of the inducing agent.

T.ni, S.frugiperda and D.melanogaster cells were grown in 60 mm plastic Petri dishes until they reached 50% confluency. Culture medium was removed and varying concentrations of IDUR, BDUR and Caffeine in 4 ml of culture medium were added. Cells were incubated at 28°C overnight in the dark.

Culture medium was then removed, 4 ml/dish of fresh culture medium added, and the cells subsequently incubated at 28°C in the dark over a period of 7 days.

At the end of this period protein concentration per plate was determined for D.melanogaster cells and cell number for T.ni and S.frugiperda cells.

Protein concentration was determined by the method of Lowry et al., (1951). The method is outlined in the legend to Figure 10.

Results of the cytotoxicity experiment are shown in Figure 10A.

It can be seen that, in all three cell lines, at IDUR and BDUR concentration in excess of 20 μ g/ml there was a cytotoxic effect of the inducing agent. This effect also occurred at Caffeine concentration above 1.5 mM.

It was determined to use 20 μ /ml concentrations for IDUR and BDUR when used as inducer and 1.5 mM for Caffeine. These concentrations were chosen as the highest concentration it was possible to use for optimal chances of induction with acceptable cytotoxic effects.

Table 10ACytotoxicity Testing of Inducing Agents on Drosophila melanogaster Cells.

Effect of inducing agent on protein concentration was determined 7 days post induction as described in Methods.

Concentration of Inducing Agent	protein/plate		Conc. of Caffeine	CAFFEINE
	IDUR	BDUR		
Control	392	392	Control	392
10 $\mu\text{g/ml}$	296	256	1.0 mM	220
20 $\mu\text{g/ml}$	232	248	1.5 mM	120
50 $\mu\text{g/ml}$	204	96	5.0 mM	5
80 $\mu\text{g/ml}$	108	5	10.0 mM	5
100 $\mu\text{g/ml}$	88	5	20.0 mM	5

Cytotoxicity Testing of Inducing Agents on T.ni cells.

Effect of inducing agent on protein concentration was determined 7 days post induction as described in Methods.

Concentration of Inducing Agent	protein/plate		Conc. of Caffeine	CAFFEINE
	IDUR	BDUR		
Control	13.5×10^5			
10 $\mu\text{g/ml}$	6×10^5	-	1.0 mM	6.5×10^5
20 $\mu\text{g/ml}$	4×10^5	11.5×10^5	1.5 mM	19×10^5
50 $\mu\text{g/ml}$	-	-	5.0 mM	13×10^5
80 $\mu\text{g/ml}$	-	2.5×10^5	10.0 mM	-
100 $\mu\text{g/ml}$	-	-	20.0 mM	-

Cytotoxicity Testing of Inducing Agents on S.frugiperda cells.

Effect of inducing agent on protein concentration was determined 7 days post induction as described in Methods.

Concentration of Inducing Agent	protein/plate		Conc. of Caffeine	CAFFEINE
	IDUR	BDUR		
Control	22×10^5	18×10^5		12.5×10^5
10 $\mu\text{g/ml}$	19×10^5	12.5×10^5	1.0 mM	13.5×10^5
20 $\mu\text{g/ml}$	20×10^5	8.5×10^5	1.5 mM	8×10^5
50 $\mu\text{g/ml}$	14.6×10^5	2.3×10^5	3.0 mM	3.2×10^5
70 $\mu\text{g/ml}$	10.5×10^5	1×10^5	5.0 mM	1×10^5

T.ni, S.frugiperda and D.melanogaster were induced with the three inducing agents at the concentration described above, (20 μ M for IDUR and DDUR and 1.5 mM for Caffeine) by the method described earlier.

Fluids were harvested from induced cells every day for 7 days for T.ni and S.frugiperda and 17 days for D.melanogaster cells.

Cell fluids from T.ni and S.frugiperda were assayed for rAdT utilizing activity as described earlier. Cell fluids from D.melanogaster cells were also assayed for rAdT utilization together with concentration of protein per plate.

Results for T.ni and S.frugiperda are shown below, data for D.melanogaster cells are shown in Results.

As can be seen from the tables below poly(rA).oligo(dT)₁₂₋₁₈ utilization by induced T.ni and S.frugiperda cells was very low after inducer was washed off (Day 1) in all cases. The fluid above D.melanogaster cells (see later) post induction with, for example, IDUR incorporated 15,000 cpm per assay, (pre-correction for protein/plate). We therefore determined to proceed with our experiments using D.melanogaster cells.

Poly(rA).oligo(dT) 12-18 utilization by induced T.ni cell fluids

Assays were carried out as described in Methods. Counts shown were cpm/ 10^4 cells/plate.

TIME (Days)	rAdT utilization		
	IDUR	BDUR	CAFFEINE
0 (inducer on)	3500	3500	3500
1 (washed off)	-	9500	15000
2	3000	1000	3000
3	-	-	-
4	-	-	-
5	1380	700	1880
6	-	-	-
7	2300	1130	-

Poly(rA).oligo(dT) 12-18 utilization by induced S.frugiperda cell fluids

Assays were carried out as described in Methods. Counts shown were cpm/ 10^4 cells/plate.

TIME (Days)	rAdT utilization		
	IDUR	BDUR	CAFFEINE
0 (inducer on)	3000	3000	3000
1 (washed off)	6000	3500	5500
2	5000	4500	3500
3	-	-	-
4	-	-	-
5	170	260	290
6	-	-	-
7	410	3990	430

RESULTS

1. Survey of Invertebrate Cell Lines for Presence of Retrovirus

(a) Focus and Plaque Assays

Methods for the assay of murine sarcoma, (Hartley & Rowe, 1966) and murine leukemia viruses, (Klement *et al*, 1969; Rowe *et al*, 1970) have been described. The assays used in this work are derived from those applied to the murine system, and were performed as described in Methods. Both disrupted cells and cell culture fluids were investigated. Table 5 shows the results obtained: none of the cell lines assayed had any focus or plaque forming activity under the assay conditions employed, although virus controls were positive. This result does not totally rule out the presence of retrovirus in the invertebrate cell lines assayed. Rather it indicates that there are no retroviruses present within the invertebrate cells or culture fluids assayed, capable of inducing focus or plaque formation under the conditions suitable for murine viruses. Thus the result is not surprising since there are numerous barriers to infection which a putative insect retrovirus would have to overcome before plaque or focus formation could occur.

- i) Temperature; insect cells replicate at 28°C and therefore infection of murine cells incubated at 37°C may be impossible.
- ii) Receptors; cell surface receptors found on murine cells may be markedly different to those on invertebrate cells and the virus/cell interaction necessary for infection may not occur.
- iii) Intracellular restriction; a process may exist within murine cells to restrict the replication of any insect retrovirus capable of entering the cell. Equally there may be too few

Table 5. Results of focus and plaque assays on disrupted cells and cell fluid. Assays were performed as described in Methods. All assays were performed in duplicate.

* Disrupted cell samples; minimum cell number assayed 5×10^4 /ml
10 μ l of disrupted cell suspension used in assay.

† Cell fluids from above cells to be assayed.

TABLE 5.

<u>Cell Line</u>		<u>Foci</u> (FFU/ml)	<u>Plaques</u> (PFU/ml)
<u>Aedes aegypti</u>	C ^{MM}	0	0
" "	F ^I	0	0
<u>Anopheles stephensi</u>	C	0	0
" "	F	0	0
<u>Spedoptera littoralis</u>	C	0	0
" "	F	0	0
<u>Aedes pseudoscutellaris</u>	C	0	0
" "	F	0	0
<u>Rhipicephalus appendiculatus</u> ²⁵⁷	C	0	0
" "	F	0	0
<u>Rhipicephalus appendiculatus</u> ²⁴³	C	0	0
" "	F	0	0
<u>Triatoma infestans</u>	C	0	0
" "	F	0	0
<u>Predenia littura</u>	C	0	0
" "	F	0	0
<u>Heliothis zea</u>	C	0	0
" "	F	0	0
<u>Choristoneura fumiferana</u>	C	0	0
" "	F	0	0
<u>Spedoptera frugiperda</u>	C	0	0
" "	F	0	0
<u>Aedes albopictus</u> ^{WAR}	C	0	0
" "	F	0	0
<u>Aedes albopictus</u> ^{MM}	C	0	0
" "	F	0	0
<u>Anopheles gambiae</u>	C	0	0
" "	F	0	0
<u>Drosophila melanogaster</u>	C	0	0
" "	F	0	0
<u>Malacasoma disstria</u>	C	0	0
" "	F	0	0
<u>Carpocapsa pomonella</u>	C	0	0
" "	F	0	0
<u>Trichoplusia ni</u>	C	0	0
" "	F	0	0
KMSV/KLV	F	3.5x10 ²	4.3x10 ²

particles to detect within the limits of the assay.

This problem of biological assay will be further discussed later.

(b) Assay for Reverse Transcriptase

The polymerase activity exhibited by a virus reverse transcriptase in response to an added template.primer depends on the composition of the template.primer (Verma, 1977). In order to screen a large number of cell lines for the presence of reverse transcriptase, the RNA.DNA template.primer poly (rA).oligo(dT)₁₂₋₁₈ was utilized. This template.primer is not absolutely specific for reverse transcriptase, however, it can be used to detect RNA-directed DNA polymerases and is useful as a screening tool. This assay determines the ability of a sample to synthesize a poly(dT) chain from dTTP as directed by the poly(rA).oligo(dT) template.primer.

Assays were performed as described in Methods and the results shown in Table 6.

Five cell lines contained significant amounts rA.dT utilizing activities, while other cell lines showed low levels of incorporation, not significantly above background. Positive cell lines were taken and their rA.dT utilizing ability further analysed (see below).

2. Characterization of rA.dT Positive Cell Lines

(a) Sucrose Gradient Fractionation

The five cell lines with significant rA.dT utilization levels were then further analysed. Cells were disrupted and fractionated by sucrose gradient centrifugation. Fractions were then assayed for their ability to utilize the template primer poly (rA).oligo(dT) as described in Methods.

Table 6. Poly(rA).oligo(dT) utilization by insect cells and culture fluids. Assays were performed as described in Methods.

* Disrupted cell samples; minimum cell number assayed 5×10^4 cell/ml
Assays were performed on 10 μ l of disrupted cell suspension.

† Cell fluids from above cells to be assayed.

TABLE 6

Cell Line	Poly(rA).oligo(dT) Utilization	
	³ H-triphosphate incorporated above background (ct/min)	
<u>Aedes aegypti</u>	C*	7349
" "	F†	3046
<u>Anopheles stephensi</u>	C	0
" "	F	0
<u>Spedoptera littoralis</u>	C	190
" "	F	60
<u>Aedes pseudoscutellaris</u>	C	0
" "	F	0
<u>Rhipicephalus appendiculatus</u> ²⁵⁷	C	853
" "	F	0
<u>Rhipicephalus appendiculatus</u> ²⁴³	C	0
" "	F	0
<u>Triatoma infestans</u>	C	621
" "	F	0
<u>Predenia littura</u>	C	0
" "	F	0
<u>Heliothis zea</u>	C	0
" "	F	0
<u>Choristoneura fumiferana</u>	C	0
" "	F	0
<u>Spedoptera frugiperda</u>	C	11300
" "	F	3800
<u>Aedes albopictus</u> ^{WAR}	C	115
" "	F	0
<u>Aedes albopictus</u> ^{MM}	C	9400
" "	F	3700
<u>Anopheles gambiae</u>	C	135
" "	F	0
<u>Drosophila melanogaster</u>	C	10500
" "	F	700
<u>Malacasoma disstria</u>	C	0
" "	F	0
<u>Carpocapsa pomonella</u>	C	0
" "	F	0
<u>Trichoplusia ni</u>	C	14600
" "	F	2500
KMSV/KLV	F	4791

Aedes aegypti: Figure 11 shows that there was no detectable peak of polymerase activity within the density gradient.

Aedes albopictus^{mm}: Figure 12 illustrates the polymerase profile of disrupted Aedes albopictus^{mm} cells. Two peaks of polymerase activity were detected at densities of 1.17 and 1.21 g/ml. The peak at 1.17 g/ml is close to the published densities of C-type particles, 1.21 g/ml being similar to the density of intracisternal A-type particles (Robertson et al, 1975 and Kreuger, 1976).

Drosophila melanogaster: A peak of polymerase activity at 1.22 g/ml was detected in fractions from the gradient of disrupted D.melanogaster cells, Figure 13. This peak of activity was also at the same density as that recorded for intracisternal A-type particles.

Spodoptera frugiperda: Gradient centrifugation and fractionation of disrupted S.frugiperda cells resulted in a peak of polymerase activity at 1.22 g/ml, Figure 14.

Trichoplusia ni: Polymerase activity was detected at 1.23 g/ml, Figure 15, although this was seen as a shoulder on a descending curve.

(b) Utilization of Specific Template.Primer

Since rAdT is a non-specific template.primer, the polymerase activities which were being detected may not have been reverse transcriptase. The synthetic template.primer polymethylcytidilate-oligodeoxyguanylate (poly(rCm).oligo(dG)) has been reported to be a highly specific probe for virus reverse transcriptases (Gerard et al, 1974). This template.primer was therefore used in the standard reaction mix as described in Methods to assay the peak fraction polymerase activities. The results of these assays are shown in Table 7.

As can be seen from Table 7, of the polymerase cell lines the D.melanogaster line was the most active with the specific

Figure 11. Sucrose density centrifugation of A.aegypti disrupted cells. Approximately 20×10^6 cells were layered onto each gradient analysed.

○: Density determined by optical refraction.

●: rAdT utilizing activity.

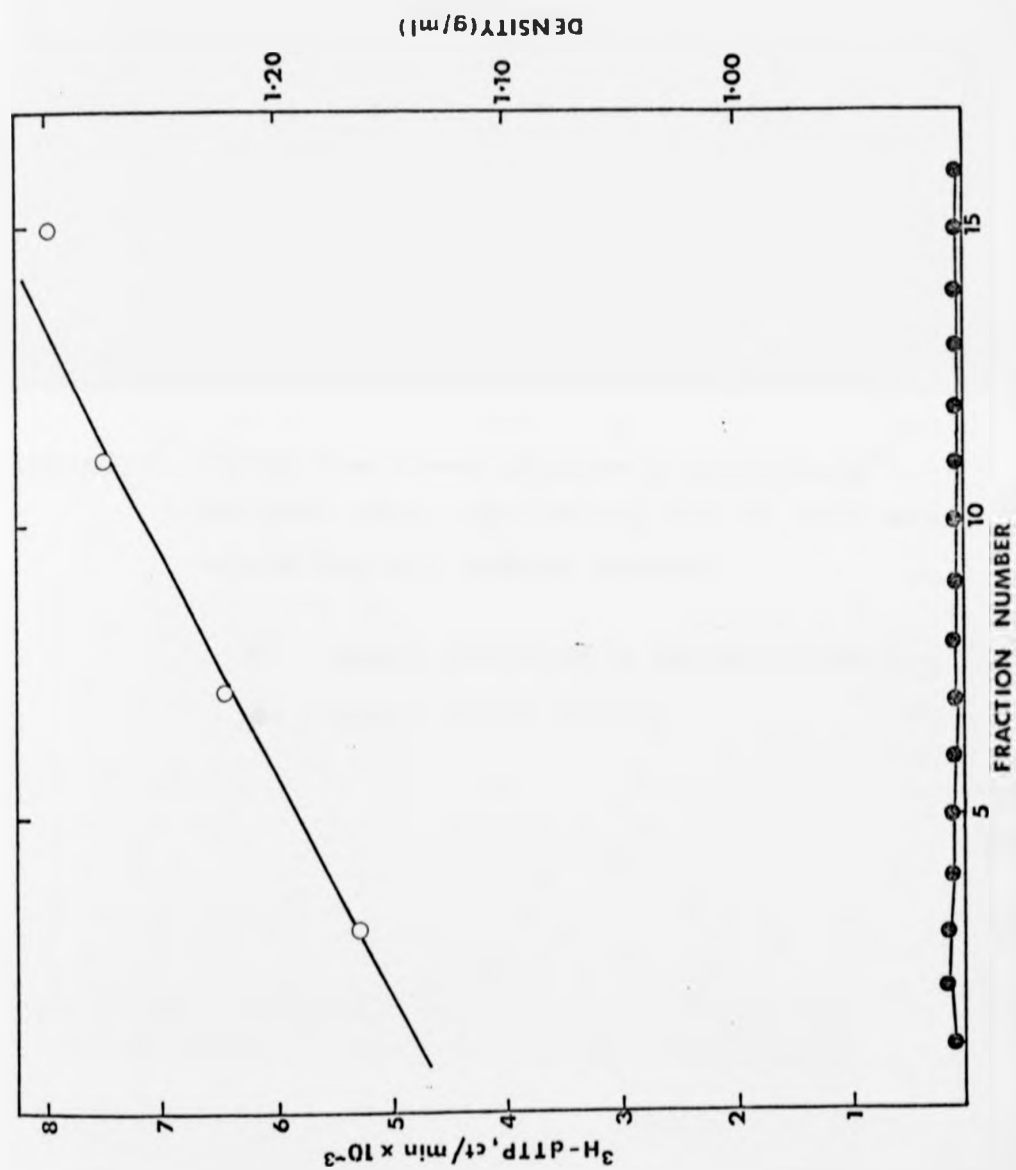


Figure 12. Sucrose density centrifugation of A.albopictus^{MM} disrupted cells. Approximately 20×10^6 cells were layered onto each gradient analysed.

- : Density determined by optical refraction.
- : rAdT utilizing activity.

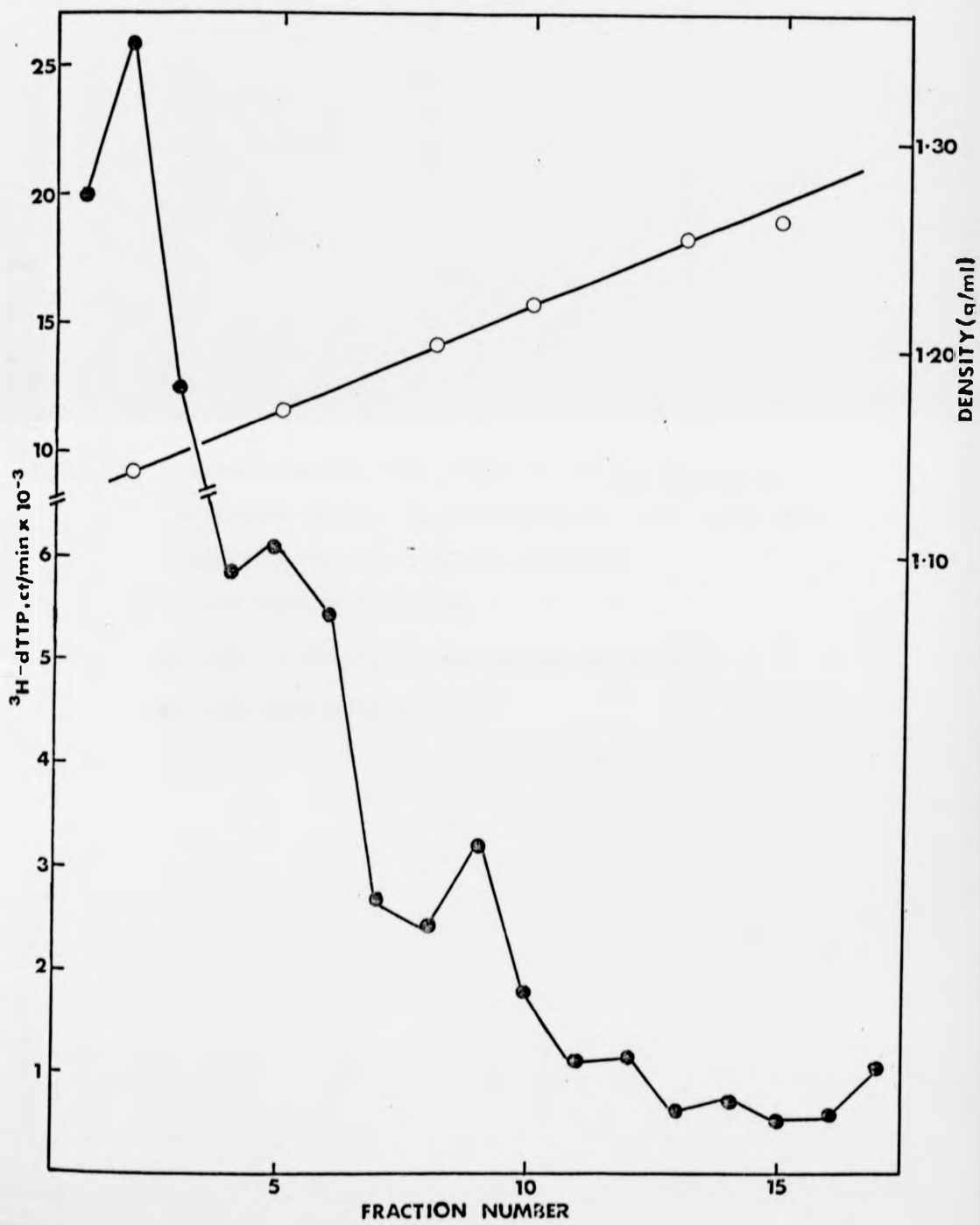


Figure 13. Sucrose density centrifugation of D.melanogaster disrupted cells. Approximately 20×10^6 cells were layered onto each gradient analysed.

LD : Low Density fractions

○: Density determined by optical refraction.

●: rAdT utilizing activity.

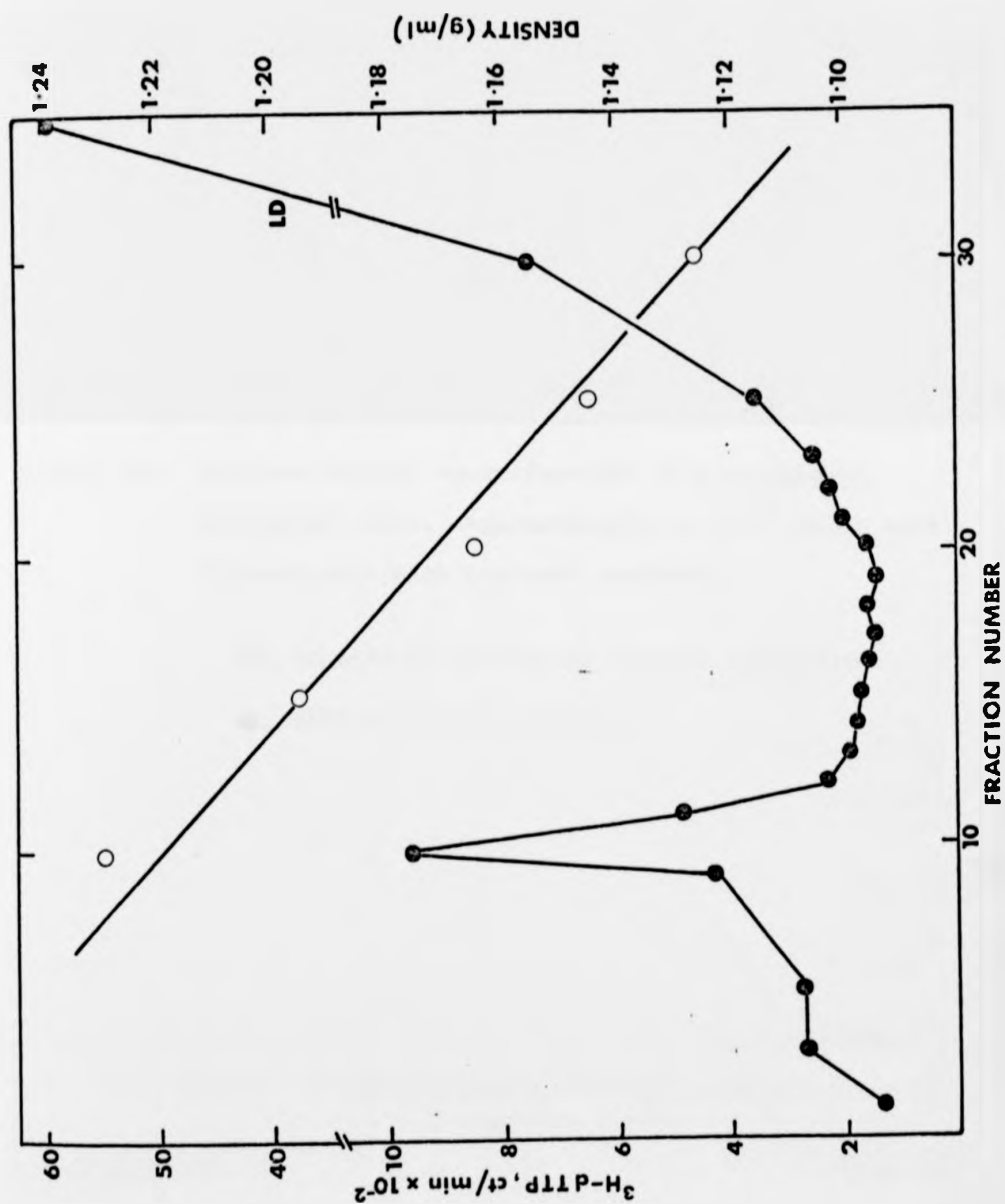


Figure 14. Sucrose density centrifugation of S.frugiperda disrupted cells. Approximately 20×10^6 cells were layered onto each gradient analysed.

○: Density determined by optical refraction.

●: rAdT utilizing activity.

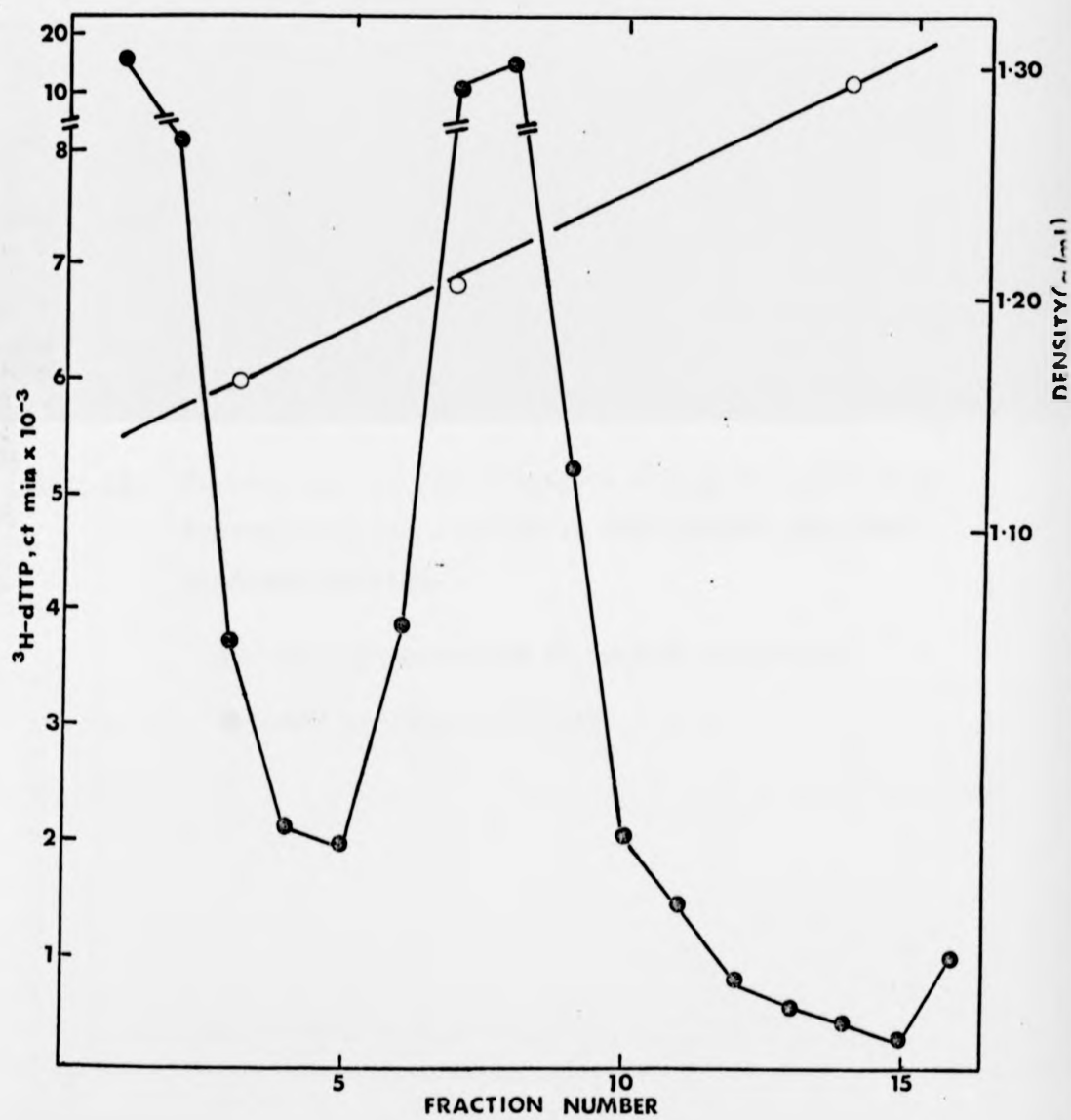


Figure 15. Sucrose density centrifugation of T.ni disrupted cells. Approximately 20×10^6 cells were layered onto each gradient analysed.

○: Density determined by optical refraction.

●: rAdT utilizing activity.

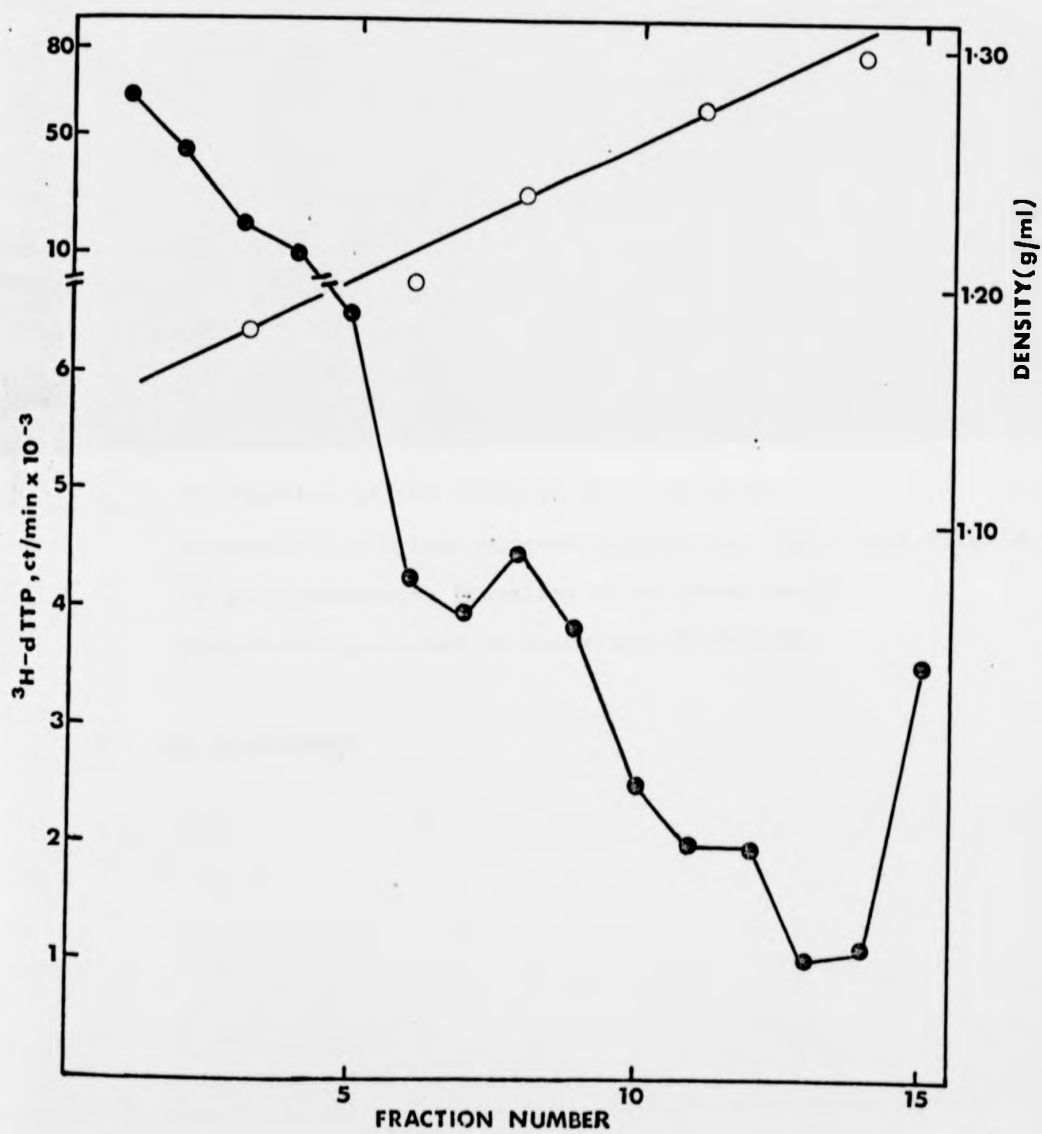


Table 7. Utilization of the specific template.primers

Polymethylcytidylate.oligodeoxyguanylate [poly(rCm).oligo(dG)]

by peak polymerase fractions from insect cells.

Assays were performed as described in Methods.

* ND : Not determined.

TABLE 7

<u>Sample</u>	<u>³H-GTP incorporated above background ct/min</u>
KMSV/KLV	1056
<u>A.aegypti</u>	ND *
<u>A.albopictus</u> ^{MM} (1.17 g/ml)	406
(1.22 g/ml)	ND
<u>D.melanogaster</u>	845
<u>S.frugiperda</u>	476
<u>T.ni</u>	153

template.primer. The next section describes experiments performed on the D.melanogaster peak polymerase fractions to further characterize the polymerase activity.

3. Characterization of Drosophila DNA polymerase Activity. Banding at 1.22 g/ml as a Viral Reverse Transcriptase

Exogenous Reactions

(1) Template primer specificities

As already stated, the polymerase activity exhibited by a virus reverse transcriptase in response to an added template.primer depends on the template.primer composition. Table 8 illustrates the template.primer response of: a) a typical murine retrovirus (KMSV), b) purified reverse transcriptase from avian myeloblastosis virus (AMV), and, c) a virus core preparation from KMSV, similar in physical characteristics to A-type particles (M. J. Byers, personal communication).

The three samples show preferential copying of template.primer in the order poly(rA).oligo(dT) > poly(rC). oligo(dG) > poly(dA). oligo(dT). The polymerase activity identified in Drosophila cells shows an identical order of template.primer preference to that of KMSV cores. Thus although the Drosophila activity is somewhat less efficient than the virus enzymes in transcribing poly(rA).oligo(dT) and poly(dA).oligo(dT) it nevertheless has relative preferences for template.primer similar to those of virus reverse transcriptases.

As a negative control, gradient fractions of 1.22 g/ml density, obtained by sucrose density gradient centrifugation of disrupted S.littoralis cells were assayed for polymerase activity. These fractions did not contain an enzyme activity capable of utilizing the RNA.DNA hybrids, although some activity was detected when the

Table 8. Template.primer specificities of Drosophila 1.22 g/ml
fraction and reverse transcriptases

* LD : Low density fraction from gradients of disrupted D.melanogaster
cells.

‡ ND : Not done.

TABLE 8

Sample	³ H-triphosphate incorporated above background (ct/min)			
	<u>Oligo(dT)</u> <u>poly(rA)</u>	<u>Oligo(dT)</u> <u>poly(dA)</u>	<u>Oligo(dG)</u> <u>poly(rC)</u>	<u>Oligo(dG)</u> <u>poly(rCm)</u>
<u>Drosophila</u> 1.22 g/ml fraction	8,357	0	2,786	845
<u>Drosophila</u> LD ^x fraction	13,153	ND [‡]	ND	0
<u>S.littoralis</u> 1.22 g/ml fraction	0	241	0	ND
KMSV	80,915	888	1,337	226
KMSV cores	15,449	478	880	ND
AMV reverse transcriptase	2,498,357	343,542	19,973	362

assay mixture contained the DNA.DNA hybrid (Table 8). The specific template.primers poly(rCm).oligo(dG) was also used to assay two virus reverse transcriptases. Both of the positive controls (the purified AMV enzyme and detergent-disrupted KMSV) can utilize the specific template.primers. As has already been shown, the 1.22 g/ml density fraction from D.melanogaster cells also has the ability to utilize the specific template.primers (Tables 7 and 8), providing further evidence that this polymerase activity is a reverse transcriptase and not a normal insect cell polymerase. Low density (LD) fractions (Figure 13) from D.melanogaster cells show no ability to utilize poly(rCm).oligo(dG) and are believed to contain cellular polymerases.

(11) Divalent Cation Requirement

Reverse transcriptases, in common with other polymerases, require divalent cation as co-factors. Most C-type retrovirus reverse transcriptases have greater activity in the presence of Mn^{2+} , as compared to Mg^{2+} ions (Green et al, 1970; Scolnick et al, 1970). This preferential utilization of Mn^{2+} ions is also shown by many intracisternal A-type particles (Yang & Wivel, 1974; Robertson et al, 1975).

The activities of the Drosophila 1.22 g/ml fraction while utilizing the poly(rA).oligo(dT) template.primers in the presence of Mn^{2+} or Mg^{2+} ions are shown in Figure 16. It can be seen that at all tested cation concentrations, more radioactive dTTP is polymerized when Mn^{2+} is the cation added, compared to when Mg^{2+} is present.

This is again consistent with the presence of a reverse transcriptase activity of viral origin in the 1.22 g/ml fraction from Drosophila cells.

As can be seen from Figure 16, the curve for Mn^{2+} concentration reaches a plateau at 1.0 mM. After this plateau, the marked increase in 3H-TTP incorporation is believed to be due to non-specific binding.

(iii) Temperature dependence

Virus reverse transcriptases generally possess a temperature optimum below that of the host cell. Experiments were carried out to determine the temperature optimum of the Drosophila 1.22 g/ml fraction whilst utilizing poly(rA).oligo(dT) as template.primers. Assays were performed as described in Methods. Results are shown in Figure 17.

The results are inconclusive as the activity appears to be insensitive to temperature between 0° and 28°C although at temperatures over 28°C there is a decrease in 3H-TTP incorporation.

Endogenous Reaction

Template.primers are useful for demonstrating the activity of reverse transcriptase either as a purified enzyme or in viral particles. However, in the latter case, a template (the virus genome) is already present and so an endogenous reaction can be detected in the absence of template.primers (Baltimore, 1970; Temin & Mizutani, 1970). In order to determine whether the activity identified in Drosophila cells was associated with a template, a polymerase assay was carried out in the absence of added template.primers. Figure 18 shows that under these conditions the 1.22 g/ml fraction from Drosophila cells was still capable of polymerizing deoxynucleotide triphosphates. This reaction reached a plateau in about 3 hours and showed considerably less incorporation than detected in the presence of poly(rA).oligo(dT). In order to determine the nature of the template associated with

Figure 16. Effect of divalent cation concentration on utilization of poly(rA).oligo(dT) by Drosophila 1.22 g/ml fraction. Assays were performed by adding varying concentrations of divalent cation to the standard reaction mix as described in Methods.

▲: Mn^{++}

△: Mg^{++}

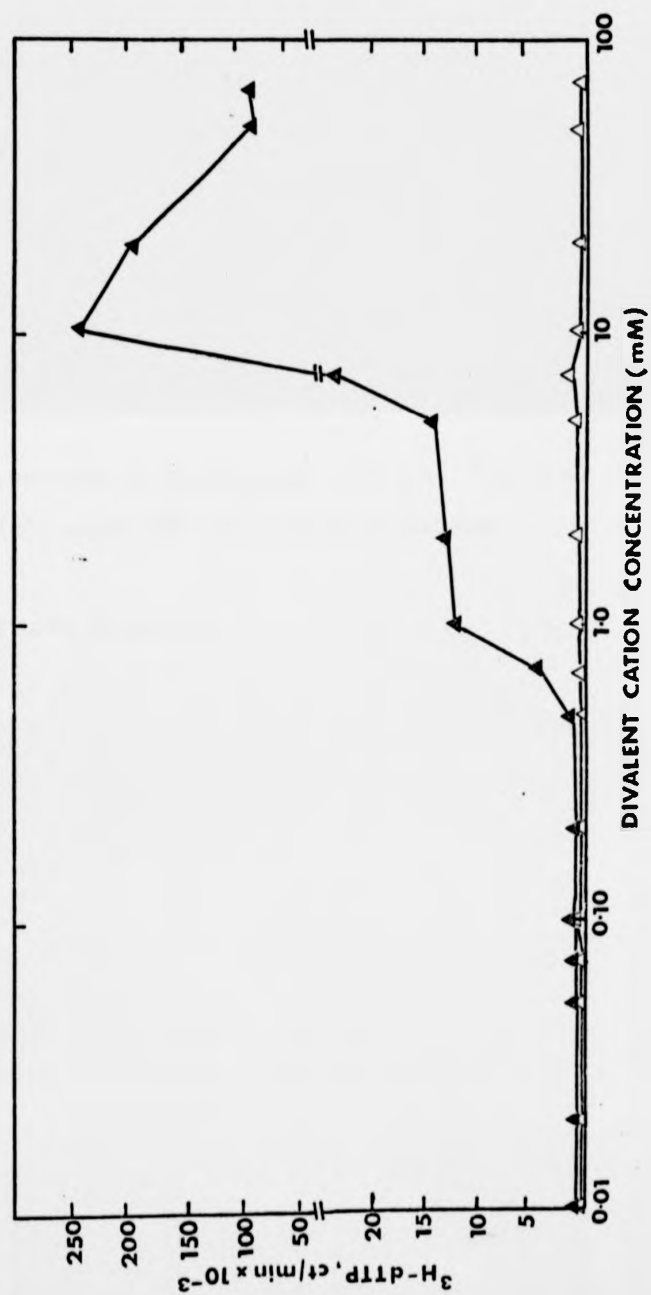
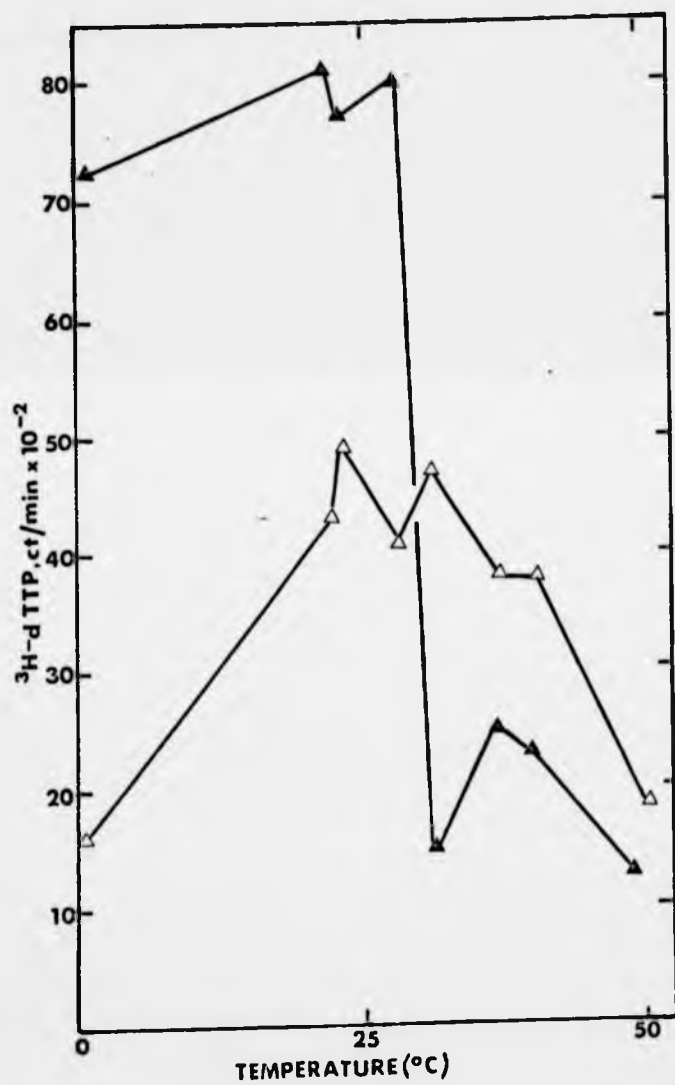


Figure 17. Temperature dependence of Drosophila 1.22 g/ml fraction
utilizing poly(rA).oligo(dT) as a template.primers.

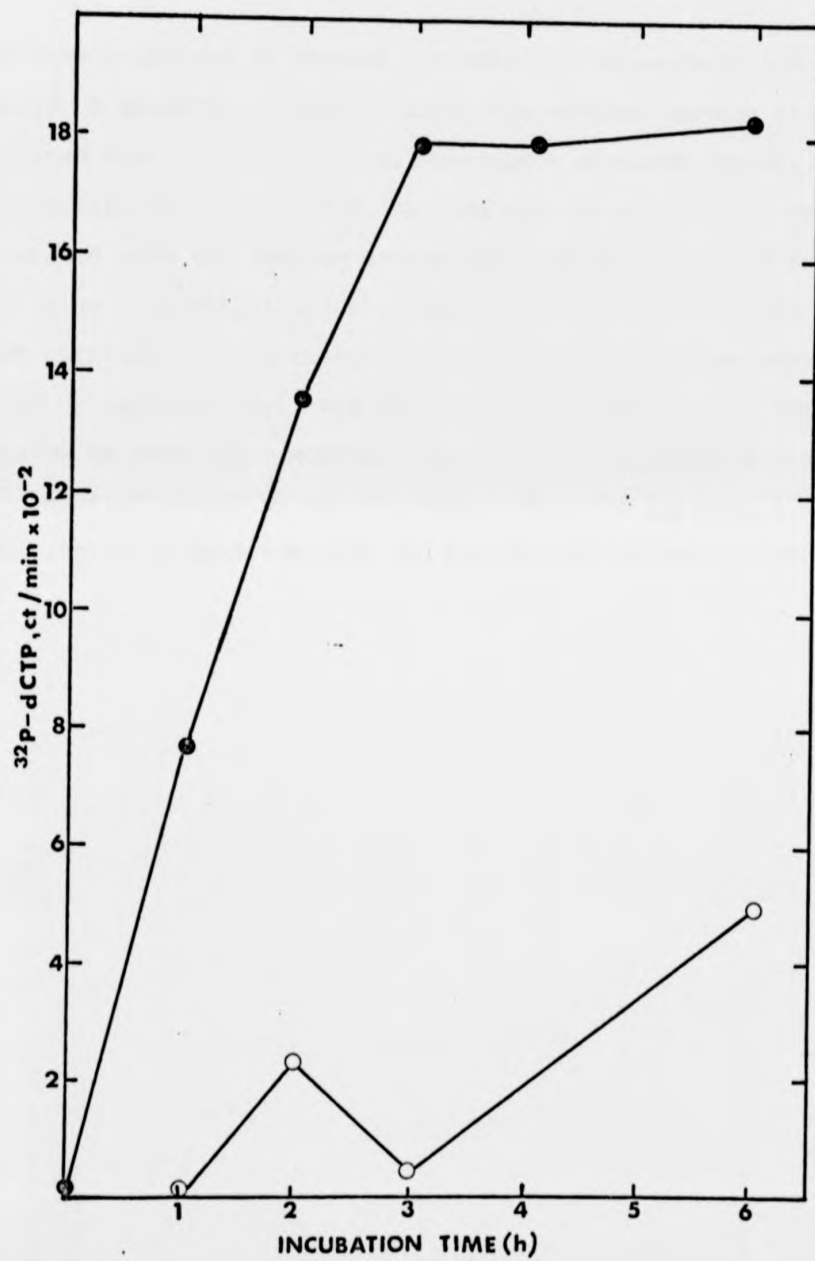
▲ Drosophila 1.22 g/ml fraction

△ KMSV



the Drosophila activity, an endogenous reverse transcriptase assay was carried out following DNase-free Rnase digestion of the 1.22 g/ml fraction. This treatment considerably reduced the endogenous polymerization (Figure 1B) suggesting that an RNA template was associated with Drosophila polymerase.

Figure 18. Polymerization of deoxynucleotide triphosphates,
measured by incorporation of α - ^{32}P -labelled deoxycytidine
5' triphosphate (dCTP), by 1.22 g/ml fraction, before
(● — ●) or after (○ — ○) RNase treatment.



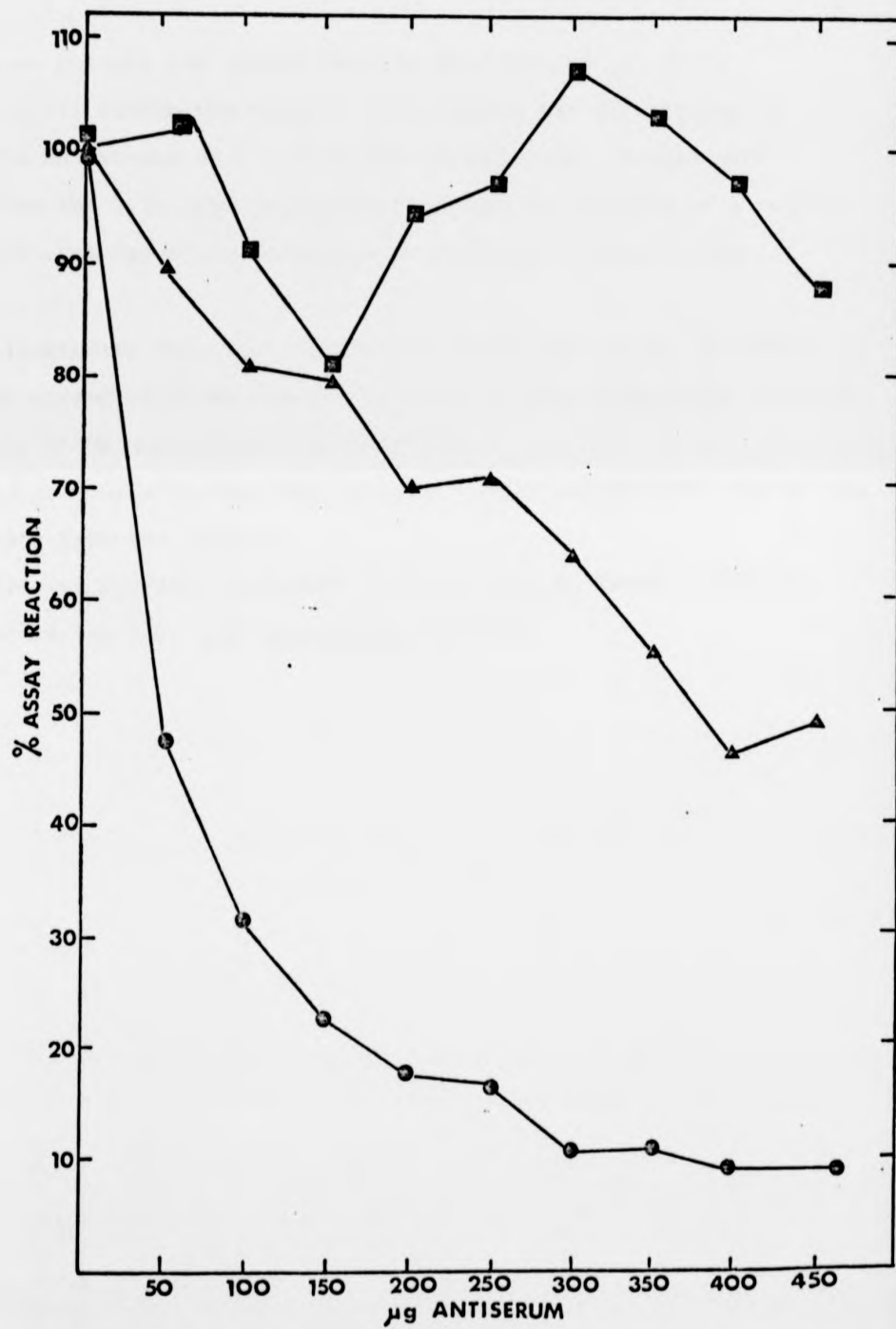
Inhibition by Antibody

Reverse transcriptases of several retroviruses cross-react immunologically. A quantity of goat antibody against AMV reverse transcriptase was obtained from Dr R E Wilsnack, Huntingdon Research Center, Brooklandville, Maryland, U S A. Varying amounts of antibody were pre-incubated with the samples before addition to a standard incubation-mix utilizing poly(rA).oligo(dT). Results are shown in Figure 19. Both AMV purified reverse transcriptase and the KMSV virus enzyme were inhibited by the antibody. The AMV enzyme was inhibited by 90%, the KMSV enzyme by over 50%. However, the 1.22 g/ml Drosophila fraction is not inhibited at all by the antibody. Thus the Drosophila polymerase is immunologically distinct from the AMV reverse transcriptase.

Figure 19. Antibody inhibition of Drosophila 1.22 g/ml fraction and reverse transcriptases while utilizing poly(rA).oligo(dT) as template.primers.

Samples were pre-incubated with varying amounts of antibody at 37°C for 15 minutes. Sample and antibody were then added to the standard poly(rA).oligo(dT) reaction mix and treated as described in Methods.

- AMV reverse transcriptase
- ▲ KMSV
- 1.22 g/ml Drosophila fraction



RNase H Activity

Retroviruses possess the enzyme RNase H (Moelling et al, 1971; Moelling, 1974) within the virion. This enzyme has the ability to degrade the RNA strand of a duplex RNA-DNA molecule. Assays were performed on the 1.22 g/ml Drosophila fraction by addition of a radio-labelled RNA.DNA hybrid, to determine if an RNase H activity was present.

Table 9 illustrates the results obtained. Over 20% of the ^3H counts in the RNA strand could be removed by the 1.22 g/ml Drosophila fraction whilst only $\sim 10\%$ were susceptible to RNase.

KMSV when incubated with the radiolabelled hybrid solubilized 15% of the total counts from the hybrid.

This experiment provides tentative evidence that an RNase H activity is present in the 1.22 g/ml Drosophila fraction.

Table 9. RNase H activity in Drosophila 1.22 g/ml fractions.

Samples were assayed as described in Methods.

RNase: pancreatic RNase A, 1 mg/ml; RNase T₁, 10³ units/ml
boiled before use for 2 minutes.

TABLE 9

SAMPLE	% TOTAL COUNTS PRECIPITABLE	
	^3H Poly (rA)	^3H poly(rA).oligo(dT)
UNTREATED	88.1 (5497 cpm)	92.5 (5602 cpm)
+ RNase	7.34	89.5
+ <u>D.melanogaster</u> 1.22 g/ml fraction	91.9	79.4
+ KMSV	78.2	85.4

DISCUSSION 1.

Survey of Invertebrate Cell Lines for Presence of Retrovirus

No evidence of plaque or focus formation was found during these experiments. This result however reflects the limitation of the experimental approach.

- (i) Under the infection conditions of the experiments, the temperature of inoculation was set at 37°C. Since we are assaying for an invertebrate virus, this temperature may be inhibitory and therefore infection may be prevented by culture conditions rather than biological restrictions. Unfortunately, however, mammalian cells do not grow at 28°C.
- (ii) Host range of the retroviruses is limited. It is therefore possible that an insufficient number of cell lines were used to screen for focus or plaque formation. Invertebrate and vertebrate cells may be very different in their membrane structure thus preventing infection by mammalian viruses.
- (iii) It is possible that there are too few retroviral particles to be detected by the assay techniques available. The use of the electron microscope is useful as a preliminary screening tool. The method is time consuming and inherently non-specific, however no biological assay is required. More specific methods of electron microscopic examination, eg immuno electron microscopy, depend upon a relatively pure antibody preparation being available. These antibodies were unavailable to us.

Perhaps the most important detection technique utilized in this work was the reverse transcriptase assay. The assay using rAdT as template primer is very sensitive, the 'amplification' of transcriptase activity being from ten to one hundred-fold. However the template primer

chosen was not absolutely specific and incorporation of radiolabel could occur with transcriptases other than reverse transcriptase, but as a preliminary screening tool, this technique proved useful.

Five cell lines contained significant amounts of intracellular rAdT utilizing activities. Lower levels of activity could be detected in cell culture fluids.

DISCUSSION 2.

Characterization of rAdT Positive Cell Lines

Sucrose gradient fractionation was used in order to determine the buoyant density of fractions with associated polymerase activities.

The distribution of intracellular organelles of invertebrate cells within sucrose density gradients is not known. Therefore it is possible that the enzyme activities detected at various buoyant densities were associated with such cellular organelles eg mitochondria.

Thus although a series of gradient fractions of varying buoyant densities in the range expected for retroviruses were isolated from the five cell lines with transcriptase activities, the nature of these activities required further investigation.

In order to characterize the enzyme activities as reverse transcriptases the specific template.primer poly rCm.oligo dG was employed.

Of the five cell lines, the Drosophila melanogaster 1.22 g/ml fraction showed the greatest utilization of this template.primer, and further characterization of this transcriptase containing subcellular fraction was carried out.

DISCUSSION 3

Characterization of *Drosophila* DNA polymerase activity

The template.primer specificities of the *Drosophila* transcriptase corresponded to those of known retroviral polymerases. They did not follow the same ratios, cpm : cpm, with the different template.primers used but the trend of utilizing rAdT over rCdG over rCmG over dAdT was the same. Since we may have been dealing with a new, invertebrate transcriptase, it was reasonable to expect some minor departures from the characteristics of the known reverse transcriptases.

Divalent cation requirements of the polymerase activity were clear, Figure 16. Manganese was the preferred divalent cation over magnesium. However, the curve obtained for manganese may be divided into two parts. Up to 5 mM divalent cation, the curve followed a reasonable scale and probably reflected the cation dependence of the transcriptase reaction. However after 5 mM divalent cation concentration, the incorporated ^3H -TTP levels rise to over 200 000 cpm, a fifteen-fold increase from the plateau level from 1 - 5 mM Mn^{++} concentration. It was possible that this was a non-specific Mn^{++} concentration effect.

Temperature range of the *Drosophila* transcriptase, shown in Figure 17, appeared to be broad. From 0°C to 26°C levels of incorporated ^3H -TTP were high, however from 28°C to 50°C levels were decreased. More points at low temperature should be obtained to clarify these results.

The validity of these results was dubious. Since we were dealing with a crude preparation it was possible that several enzyme activities were present, probably of varying temperature dependence, capable of utilizing rAdT as template.primer. The temperature profile obtained probably contained elements of all of these transcriptases.

Similar arguments may also apply to the results obtained in the divalent cation experiment. In virus particles the virus genome acts as template for the viral enzyme and enzyme activity can be detected in the absence of template.primer - the endogenous reaction. An enzyme activity was detected in the 1.22 g/ml fraction from Drosophila cells which was able to polymerize deoxynucleotide triphosphates in the absence of template.primers.

The endogenous reaction experiment reached a plateau after 3 hours and levels of incorporation of label were depressed when compared to those experiments employing template.primer. The nature of the template associated with the Drosophila activity was then determined.

RNase treatment of the sample before incubation resulted in greatly reduced incorporation of radiolabel. This result implied that an RNA template was involved in the endogenous reaction.

When antibody to AMV reverse transcriptase was added to the Drosophila 1.22 g/ml fraction, no significant decrease in rAdT utilization could be detected. While sharing characteristics with vertebrate transcriptases, the Drosophila activity is apparently not recognisably related to them as judged by the previous experiment.

When assayed for RNase H activity, the Drosophila enzyme decreased the total number of counts in a ^3H poly(rA).oligo(dT) polymer by 14.1% (ie, 13.1 out of 92.5), whereas RNase reduced the counts by 3.2% (ie. 3 out of 92.5)

A better control than KMSV would have been purified RNase H, however this was not available. The result obtained implies an RNase activity is associated with the 1.22 g/ml fraction. Attempts to increase the amounts of degradation of ^3H -poly(rA).oligo(dT) moiety failed. This may reflect the instability of the Drosophila polymerase complex. However it should be noted that a known retrovirus (KMSV) only reduced the RNA content of the DNA/RNA hybrid by 7.7% (ie. 7.1 out of 92.5)

We conclude that a virus-like entity exists in Drosophila melano-
gaster cells which shares some characteristics with, but is not closely
related to, vertebrate retroviruses.

Electron Microscopy

Intracytoplasmic A-type particles often appear as toroidal or doughnut-shaped particles within the cytoplasm of the cell, are 60 to 70 nm in diameter and of 1.26 to 1.29 g/ml density (Smith & Wivel, 1972; Tanaka et al, 1972). Intracisternal A-type particles enter the cisternae of the endoplasmic reticulum by budding from the cytoplasm and remain within them. These latter particles range in size from 70 to 100 nm in diameter (Wong-Staal et al, 1975; Robertson et al, 1976). Intracisternal particles often appear to possess a double shell, although two shells cannot always be distinguished.

Before presenting the results of electron microscopy on the Drosophila cell line we would like to discuss the history of the line.

The Drosophila cell line available to us was provided by Dr. P. Scotti of DSIR, Auckland, New Zealand to the Unit of Intertebrate Virology in Oxford. The cell line provided was Schneider's line 1 (DL 1), (Schneider, 1972), which Dr. Scotti obtained from Dr. T. Grace, (Scotti, personal communication).

After rescuing the D melanogaster line from liquid nitrogen experiments were always carried on cells which had not been passaged more than fifteen times.

Electron microscopic examination was carried out in Oxford where no RNA tumour viruses are used, thus reducing the likelihood of contamination of the cell line by these viruses. Dr. Scotti also stated he believed the line he supplied to Oxford to be free of small RNA viruses (Scotti, personal communication).

A large number of E.M. grids containing sections of Drosophila cells were examined, (at least 50 grids) and 90% of these grids contained the particles described below.

Electron micrographs of our D melanogaster cell line show toroidal shaped particles present in the cytoplasm (Figure 20). These particles are of 40 nm diameter and have the typical morphology of intracytoplasmic

A-type particles (Smith & Wivel, 1972; Tanaka et al, 1972).

Electron microscopic examination of 1.22 g/ml fractions was also carried out, as described in the legend to Figure 20. This revealed virus-like structures (Figure 20(c)) not present on control grids and of similar size to the toroidal particles shown in Figures 20(a) and (b). Electron microscopy was also carried out on all other invertebrate cell lines which showed significant levels of poly (rA) and oligo (dT)₁₂₋₁₈ utilization. None of the fields examined, (at least 20 grids for each cell line), showed any unusual intracellular forms similar to those observed in the Drosophila melanogaster cell line.

This observation also indicates that the calf serum used was not a source of viral contamination since D.melanogaster and those cell lines with no observed unusual intracellular entities were grown in medium which utilized calf serum.

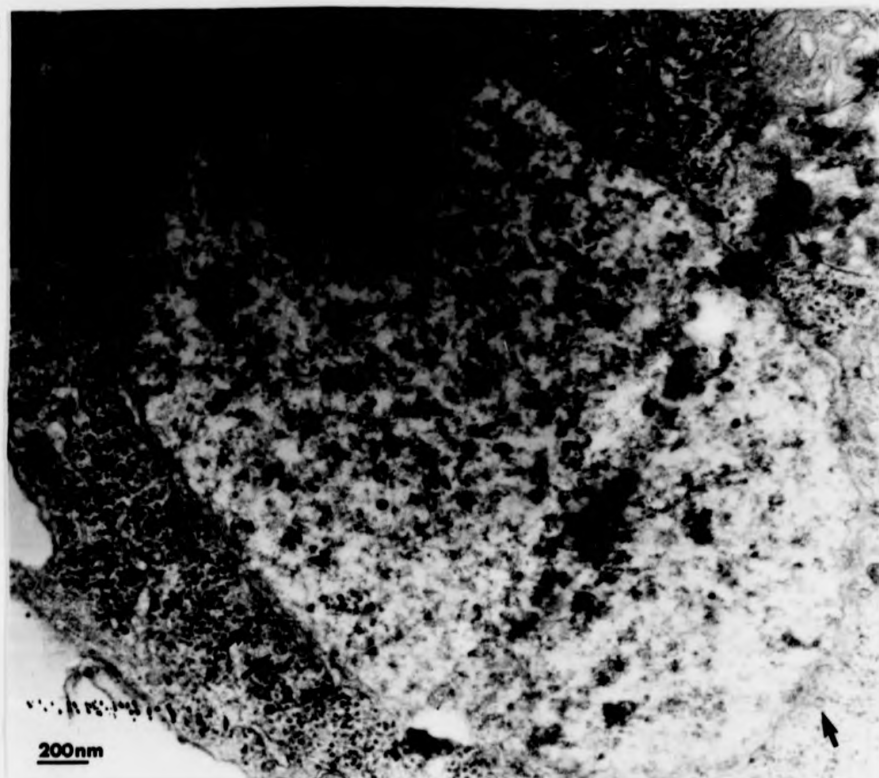
Figure 20. (a) Toroidal bodies in Drosophila cells

(b) Electron micrograph of toroidal particles at higher magnification. Toroids have a diameter of 40 nm.

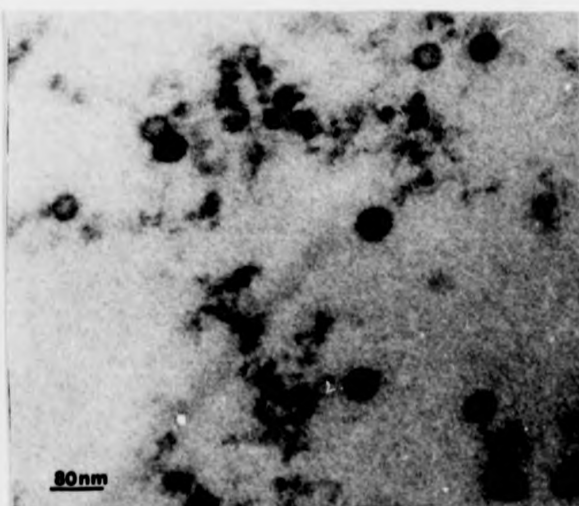
(c) Electron micrograph of the 1.22 g/ml fraction.

Peak enzyme fractions were pooled, diluted two-fold with PBS and centrifuged at 110,000 g overnight. After centrifugation, the supernatant fluid was discarded and the pellet taken up in 100 of PBS. Samples were placed on electron microscope grids coated with Formvar and were stained with 0.5% phosphotungstate. Electron microscopic examination was carried out as described in Methods.

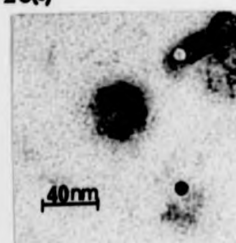
20(a)



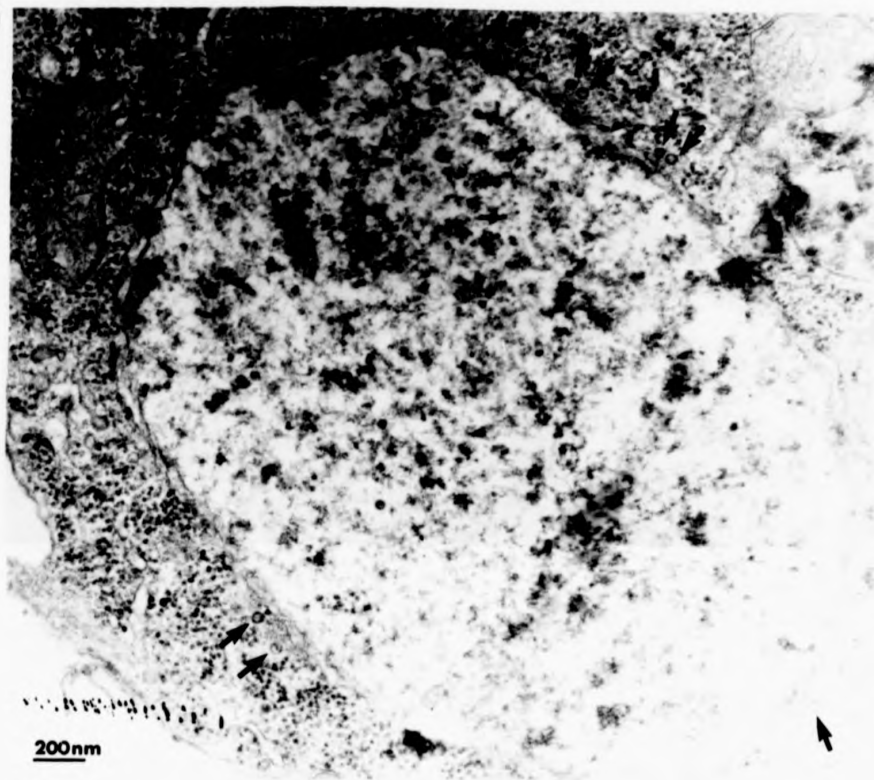
20(b)



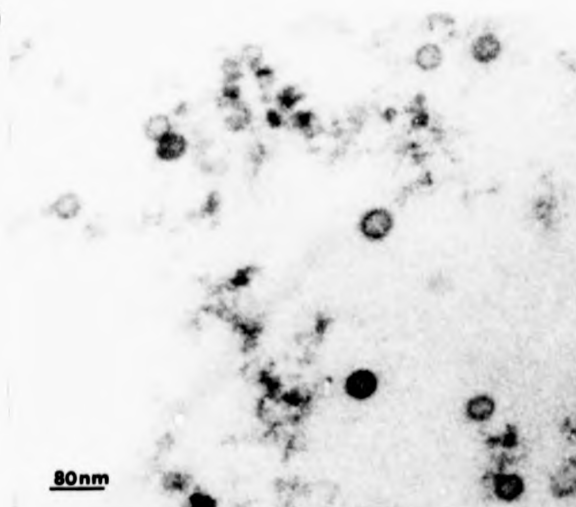
20(c)



20(a)



20(b)



20(c)



DISCUSSION 4

Electron microscopy

Unclassified virus-like particles have been observed previously in various Drosophila tissues by electron microscopy (Akai et al, 1967; Philpott et al, 1969; Felluga et al, 1971).

Particles of 36-42 nm diameter were most frequently observed and their presence did not appear to result in any cytopathic changes. Larger particles which were of 50-60 nm diameter were also observed.

Gateff, (1978), has reported that putative virus particles of many size classes occur in all malignant neoplasms of Drosophila and following cell ageing or cell damage increased numbers of particles were observed.

The toroidal particles shown in Figure 20 bear a morphological resemblance to the murine intracytoplasmic A-type particles previously reported, (Smith and Wivel, 1972; Tanaka et al, 1972). However their diameter was lower than that for either intracytoplasmic or intracisternal A-type particles.

Contamination of the cell line by tumour viruses held at Warwick was unlikely since most of the electron microscopy was carried out at Oxford where these viruses are not used.

Possible viral contamination from calf serum used in invertebrate cell culture medium was also unlikely as virus-like entities were only observed in Drosophila cell culture.

There is no evidence that the particles observed were associated with the transcriptase activity isolated from Drosophila cells. However when 1.22 g/ml density fractions were centrifuged at high speed and visualized under the electron microscope 40 nm icosahedral particles were observed. (Figure 20(c)).

Thus our Drosophila melanogaster cell line appears to contain intracellular retrovirus-like particles which bear morphological similarities to those observed in murine and avian cells.

Demonstration of RNA in the 1.22 g/ml Fraction from D. melanogaster Cells

D.melanogaster cells were labelled with ^3H -uridine as outlined in Methods. The radioactive cells were disrupted and fractionated on sucrose gradients as previously described. Figure 21 shows the distribution of radioactivity in the fractions obtained. A discrete peak of radioactive RNA was found in the 1.22 g/ml fraction which also contains the polymerase activity (Figure 21). This was clearly resolved from the large amounts of radioactivity found at the top of the gradient which presumably represents various forms of cellular RNA. The 1.22 g/ml fraction therefore meets the requirements of the Spiegelmann simultaneous detection test in containing both RNA and reverse transcriptase.

Characterization of RNA from 1.22 g/ml Fraction of D.melanogaster Cells

Uridine-labelled material was extracted from a radioactive 1.22 g/ml fraction prepared as described above and was shown to be susceptible to degradation by alkali and RNase. The preparative procedure involved hybridization to an oligo(dT) column. This implies the presence of poly(rA) sequences in the radioactive RNA.

Murine intracisternal A-type particles have been shown to possess a high molecular weight RNA species of 60-70S (Yang & Wivel, 1973; Robertson *et al*, 1975; Krueger, 1976), which can be dissociated by heating into smaller molecular weight components (Krueger, 1976).

Figure 22 shows a sucrose density gradient analysis of the RNA prepared from the Drosophila 1.22 g/ml fractions. A heterogeneous profile was obtained as is often the case, with known retroviruses (Vogt & Hu, 1977; Coffin, 1979). However, one major peak of activity, arrowed, represented

Figure 21. A 20-50% sucrose/STE density gradient of disrupted
³H-uridine-labelled Drosophila cells.

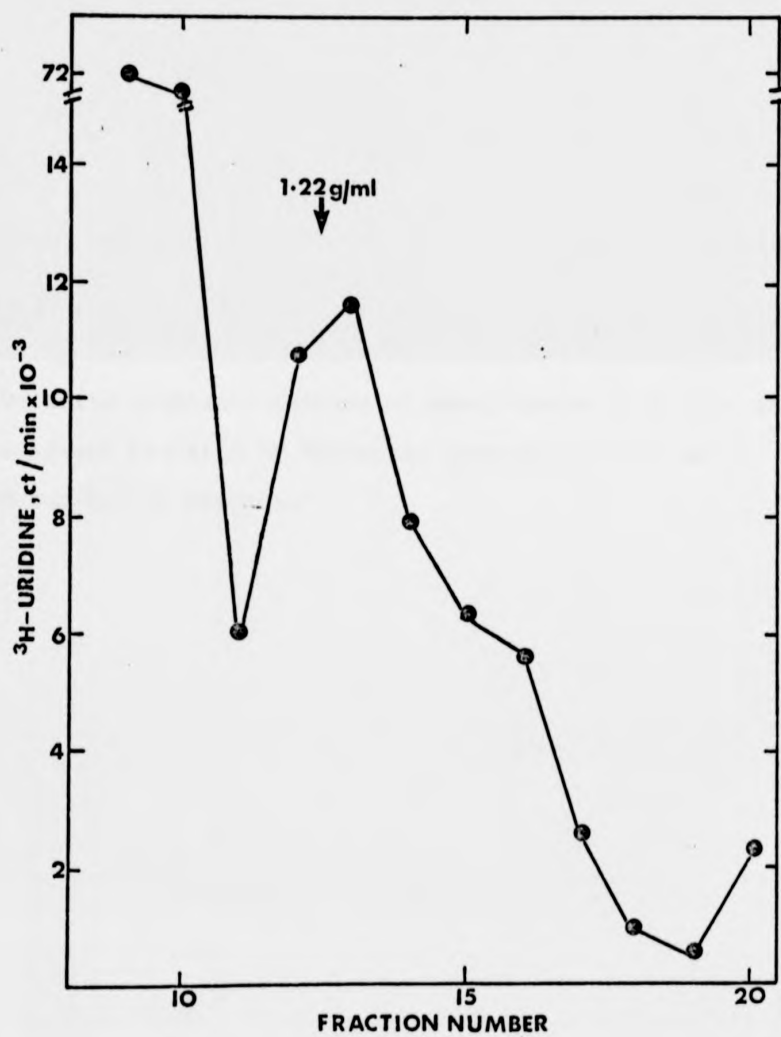
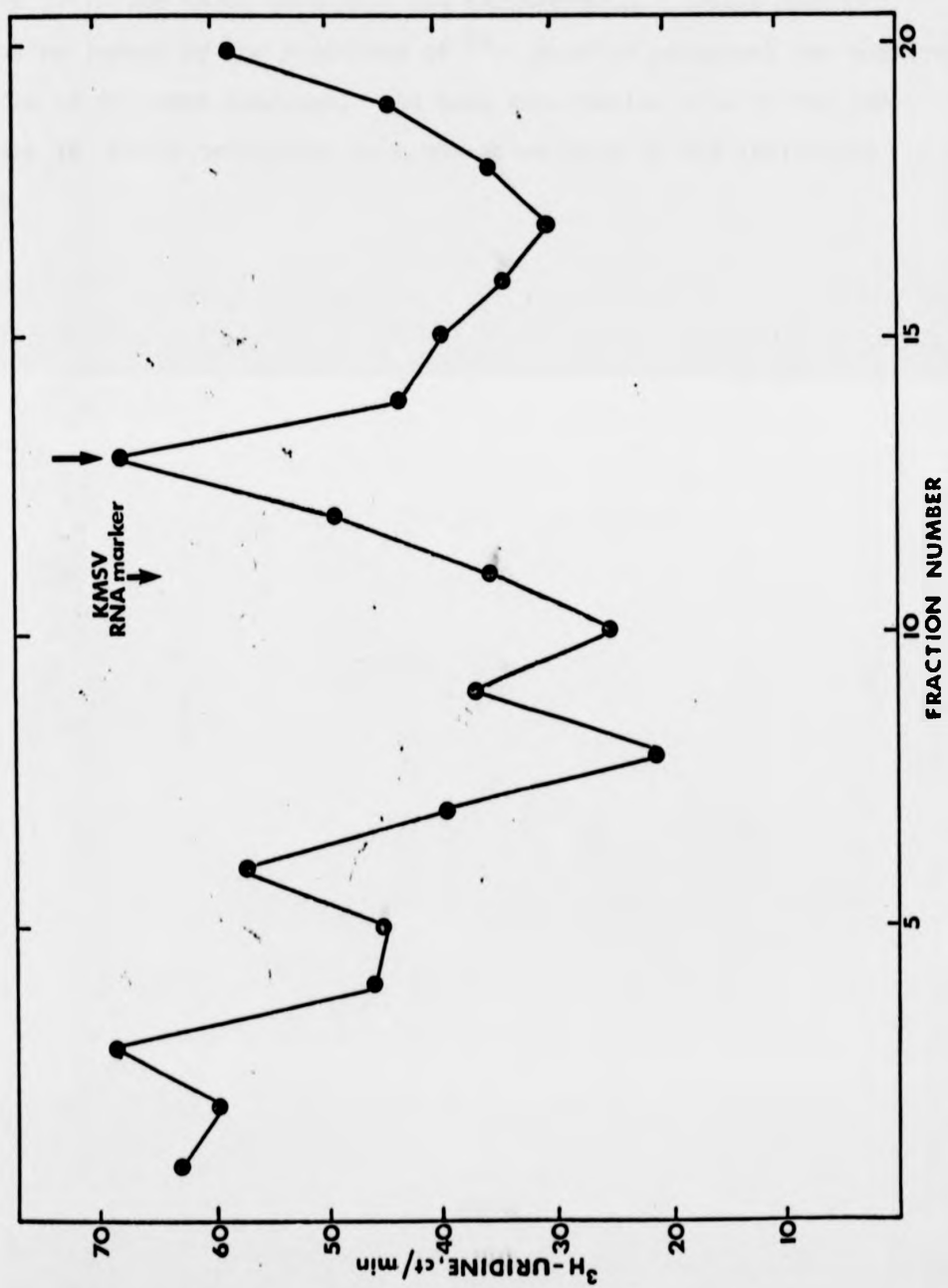
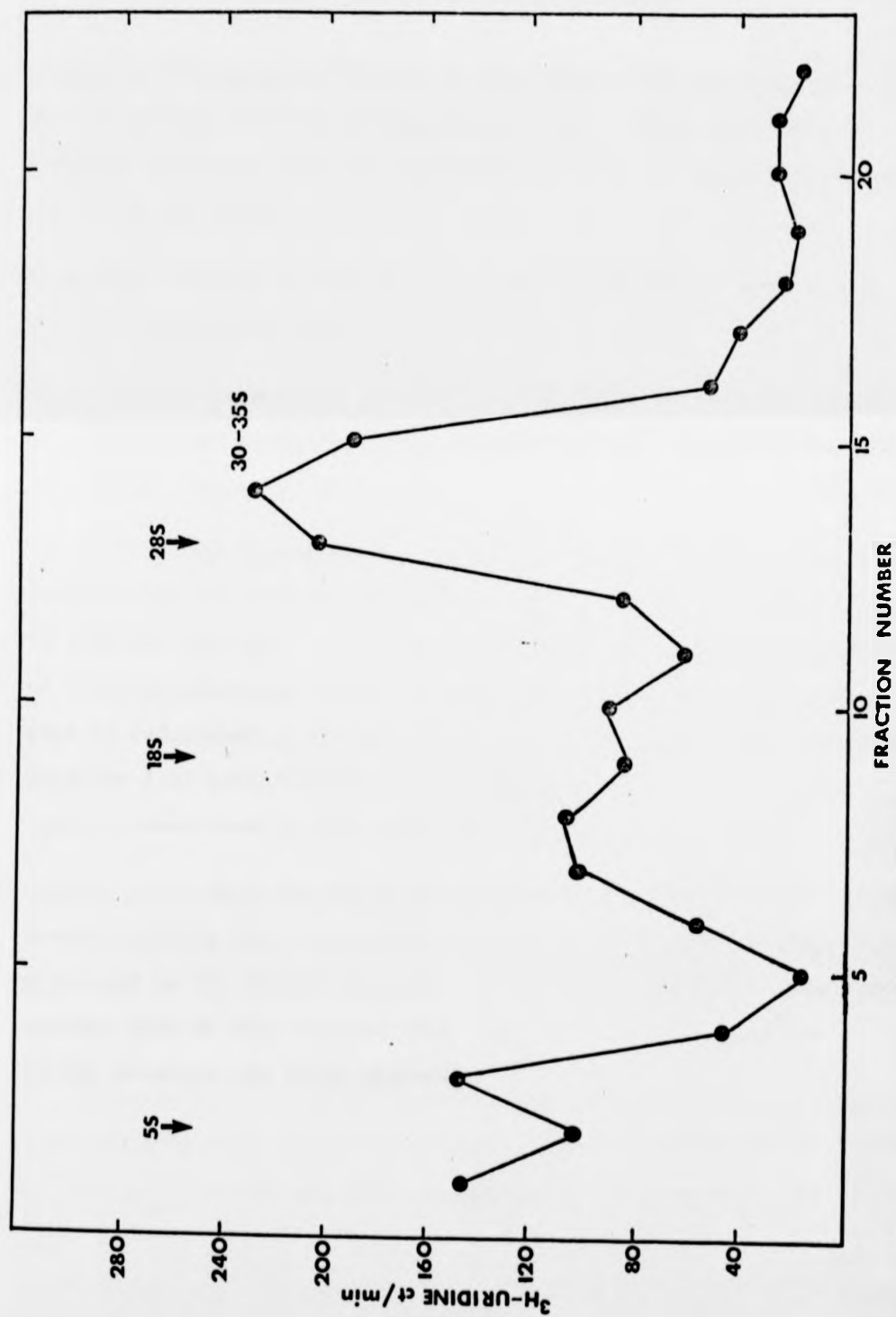


Figure 22. Velocity gradient analysis of RNA prepared from 1.22 g/ml gradient fraction of disrupted Drosophila cells as described in Methods.



a large RNA molecule since it sedimented slightly ahead of KMSV 60-70S RNA included as a marker. Heat denaturation of the RNA sample prior to gradient analysis resulted in the disappearance of this large RNA (Figure 23). The major component now sedimented as a broad peak at 30-35S, as judged by the positions of ^{14}C -labelled ribosomal RNA markers included in the same gradient. The heat degradation of a 60-70S RNA molecule to 30-35S molecules is a characteristic of the retrovirus genome.

Figure 23. Velocity gradient analysis of heat-dissociated RNA prepared from 1.22 g/ml gradient fractions of disrupted Drosophila cells as described in Methods.



DISCUSSION 5

RNA in the 1.22 g/ml Fraction from *D.melanogaster*

A peak of incorporated ^3H -uridine possessed a density coincident with the polymerase activity in *Drosophila* cells. This radiolabelled material contained poly (A) sequences and was susceptible to degradation by alkali and RNase.

From these results it appeared that the radiolabelled moiety was poly (A) containing RNA.

When analysed on sucrose gradients a high molecular weight species was observed of slightly higher molecular weight than KMSV 60-70S RNA, Figure 22.

Since it proved impossible to isolate sufficient '60-70S' 1.22 g/ml fraction RNA for heat denaturation an RNA sample was denatured prior to gradient analysis. This treatment resulted in the disappearance of the high molecular weight species and the appearance of a broad peak of radiolabel at 30-35S, Figure 23. This result was consistent with the 1.22 g/ml fraction from *Drosophila* cells containing an intracellular form of retroviral particle.

However while this was the most likely interpretation, there was no direct evidence that the 60-70S molecule was the species being denatured to the 30-35S species. It was possible that a moiety from another part of the gradient gave rise to the species and the 60-70S molecule was being degraded.

Induction of Drosophila Cells

The results so far indicate the presence of an intracellular retrovirus-like entity in Drosophila. Vertebrate cells may be 'induced' to release endogenous retroviruses by treatment with a variety of chemicals, particularly the halogenated pyrimidines.

In an effort to isolate a genuine extracellular virus particle, Drosophila cells were treated with three 'inducing' agents, iododeoxyuridine(IDUR), bromodeoxyuridine(BDUR), and caffeine. Induction was carried out as described in Methods. Experiments were carried out to determine the optimum concentration of inducer to be used. Halogenated pyrimidines have a cytotoxic effect in mammalian tissue culture systems at certain concentrations. No information was available on the invertebrate system, therefore the effects of inducer concentration on cell number and protein concentration were determined as described in Methods, data not shown.

Reverse transcriptase assays were performed on the induced cell culture fluids and the number of counts incorporated into poly(rA).oligo(dT) polymers related to the total protein on the Petri dish.

Results are shown in Figure 24. All three induction curves show a significant increase in released poly(rA).oligo(dT) utilizing activity after 3 - 6 days post-induction. IDUR appears to be the best inducer when used at 20 μ g/ml.

An isolation procedure was developed in order that this activity could be characterized. Briefly this involved harvesting induced Drosophila cell fluids every 4 hours for 2 days. After clarification of fluids by low speed centrifugation, the Drosophila 'particle' was pelleted

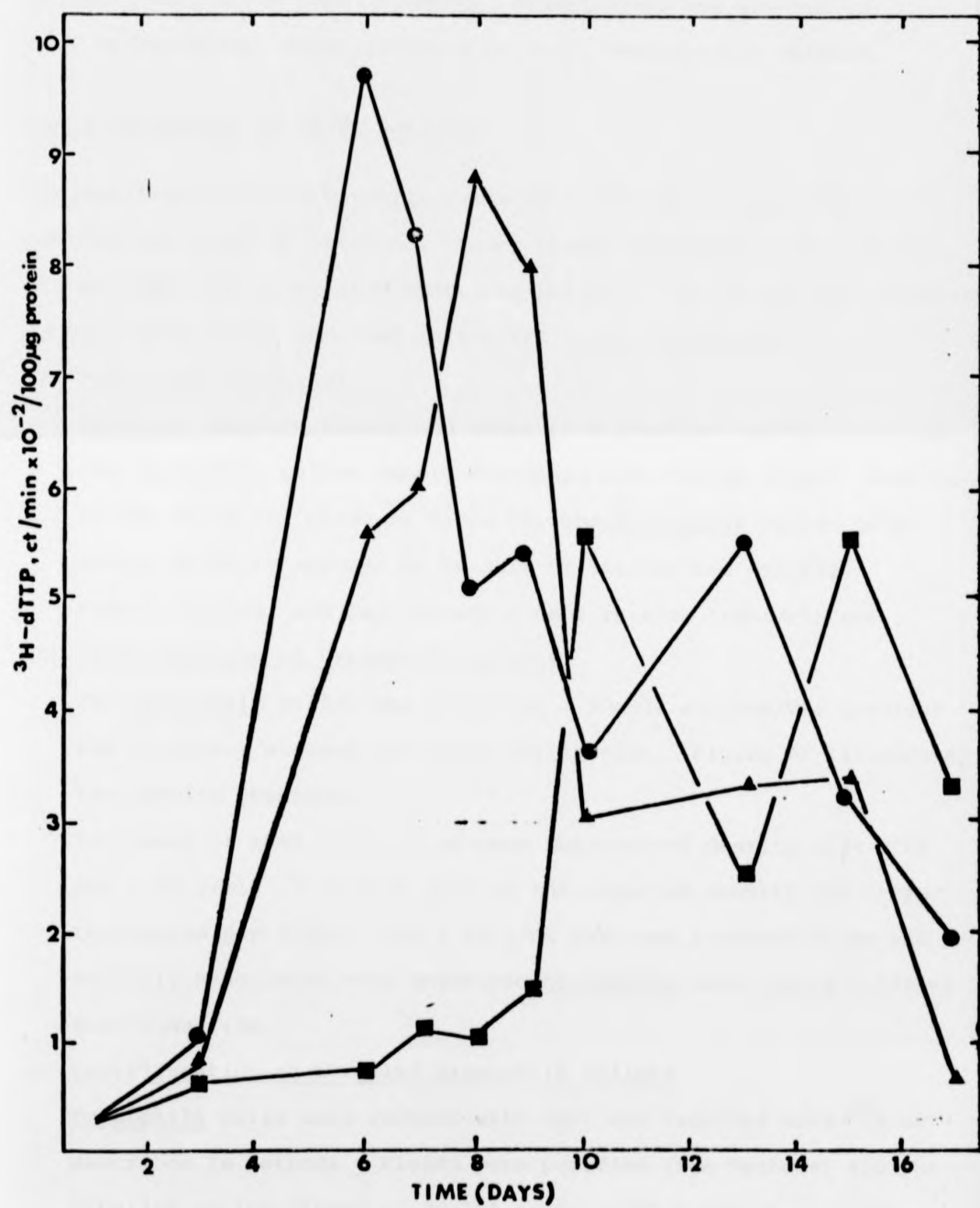
Figure 24. Induction of Drosophila cells. Cells were induced as described in Methods. Total cell protein was determined and incorporation of ^3H -TTP into poly(rA) oligo(dT) measured.

Control Drosophila cell released rAdT utilizing activity was always below 100 cpm/100 μg total cell protein.

● IDUR as inducer

▲ BDUR " "

■ Caffeine "



by a high speed centrifugation and resuspended in a small volume of buffer. This suspension was then placed on a sucrose/TNE density gradient and, after centrifugation, fractionated and assayed for rAdT utilization. This procedure is fully described in Methods.

Characterization of rAdT Activity

Induced fluids from Drosophila cells were treated as described in Methods and a pellet obtained. Under these conditions in the murine system, RMSV and associated membranes pellet. The pellet was resuspended in Tris/EDTA buffer and used in the following experiments.

(a) Poly (rCm).oligo(dG)

Specific template.primer was added to a standard reaction mix and the Drosophila pellet sample disrupted with Triton X-100. Results of the assay are shown in Table 10. The Drosophila pellet polymerase activity appears to be able to utilize the specific template.primer and may therefore be a reverse transcriptase.

(b) Centrifugation of Drosophila Pellets

The Drosophila peller was placed on a 20-50% sucrose/TNE gradient and fractions assayed for rAdT utilization. Figure 25 illustrates the results obtained.

Two peaks of rAdT utilization were observed of density 1.14 g/ml and 1.20 g/ml. 1.14-1.16 g/ml is the reported density for C-type retrovirus particles. The 1.20 g/ml peak was presumed to be the activity associated with uninduced Drosophila cells being retained post-induction.

(c) Centrifugation of Labelled Drosophila Pellets

Drosophila cells were induced with IDUR and labelled with ^{32}P as described in Methods. Fluids were purified (see Methods) and the labelled pellet placed on 20-50% scurose/TNE gradient as above. Fractions were assayed for ^{32}P activity and results are presented in Figure 26. Two peaks of activity are obtained of equivalent

Table 10. Template.primer utilization of induced Drosophila pellet.
Assays were as in Methods.

TABLE 10.

SAMPLE	³ H-triphosphate incorporated (cpm/min)	
	poly rA.oligo dT	poly rCm.oligo dG
BLANK	77	274
<u>Drosophila</u> pellet	12330	1939

Figure 25. 20-50% sucrose/TNE gradient centrifugation of Drosophila pellet. Fractions were assayed for rA.dT utilization as described in Methods.

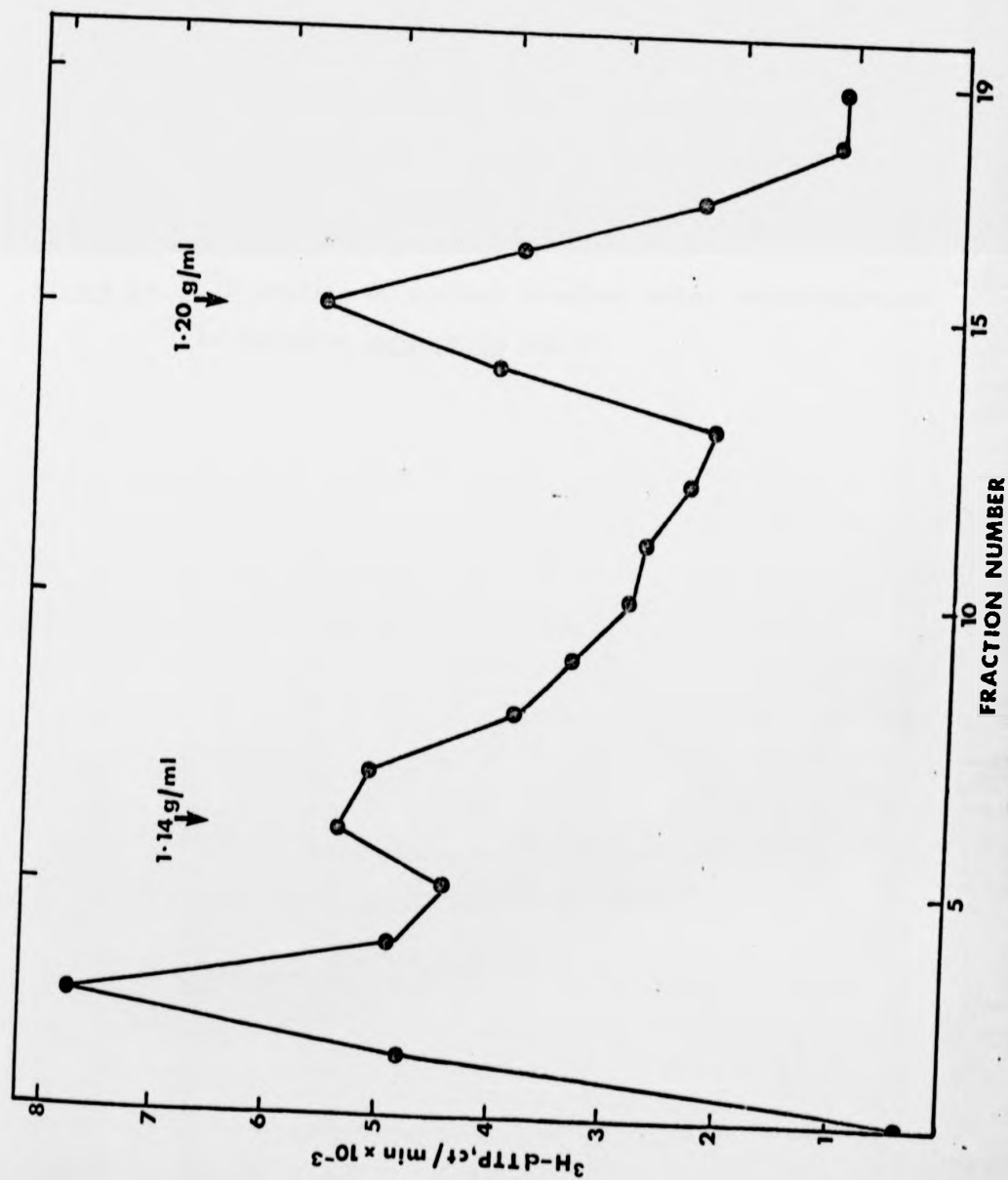
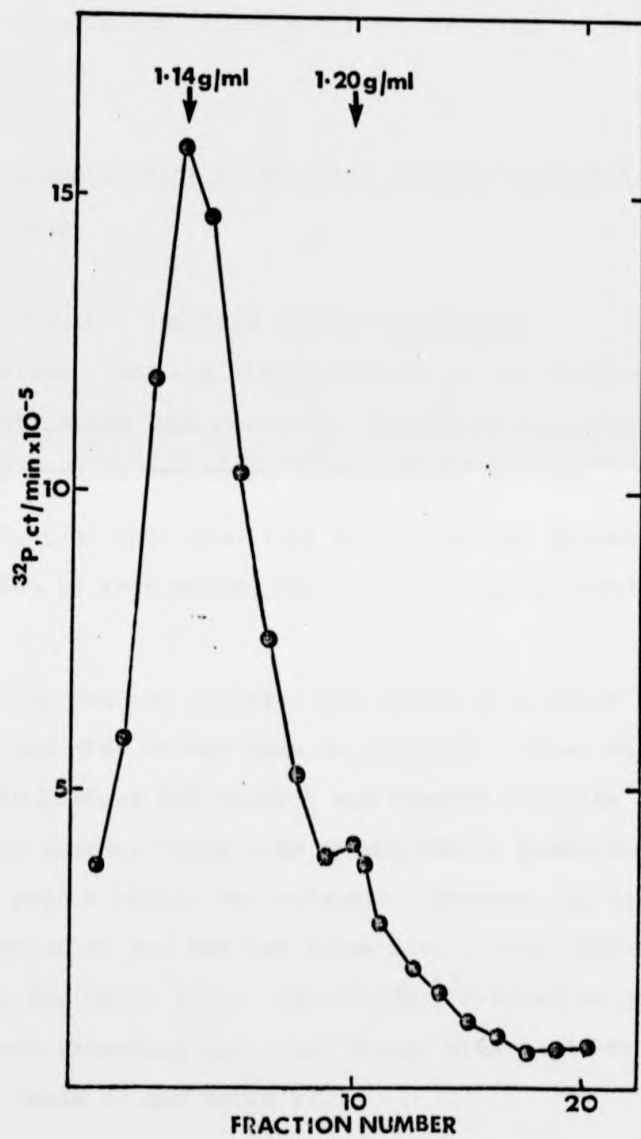


Figure 26. ^{32}P profile of sucrose gradient after centrifugation
of labelled Drosophila pellet.



density to peaks of polymerase activity, of 1.14 and 1.20 g/ml density.

In the following experiments, these two peak fractions of labelled material were utilized.

Isolation and Characterization of RNA from Induced *Drosophila* Pellet Peak Fractions

RNA was prepared from P^{32} -labelled induced *Drosophila* pellet peak fractions. RNA was then placed on 17-35% sucrose/TLES gradients and centrifuged (see Methods). Fractions were assayed for radioactivity and results are shown in Figures 27 and 28.

Both 1.14 and 1.20 g/ml peak fractions contain an RNA sedimenting at a position occupied by KMSV marker RNA of 60-70S run on parallel gradients.

The 1.14 g/ml 60-70S RNA was isolated and tested with RNase and DNase. The isolated RNA was then passed down an oligo(dT) column as described in Methods. Table 11 shows the binding and elution profiles of the RNA placed on this column. Only ~6% of the RNA is bound and therefore has a stretch of poly A within the molecule. However, it is possible that some degradation of the RNA has taken place, thus reducing the quantity bound to the oligo (dT). The 60-70S ^{32}P -labelled molecule from 1.20 g/ml peak fractions was also tested with nucleases, results are presented in Table 12 and Table 13.

As can be seen, both 60-70S moieties are relatively RNase sensitive and DNA insensitive.

1.14 g/ml peak fraction RNA was then heat denatured prior to gradient analysis. The result of such treatment was the disappearance of the large molecular weight material and subsequent appearance of label

Figure 27. Velocity gradient analysis of 1.14 g/ml fraction
P³² labelled material as described in Methods.

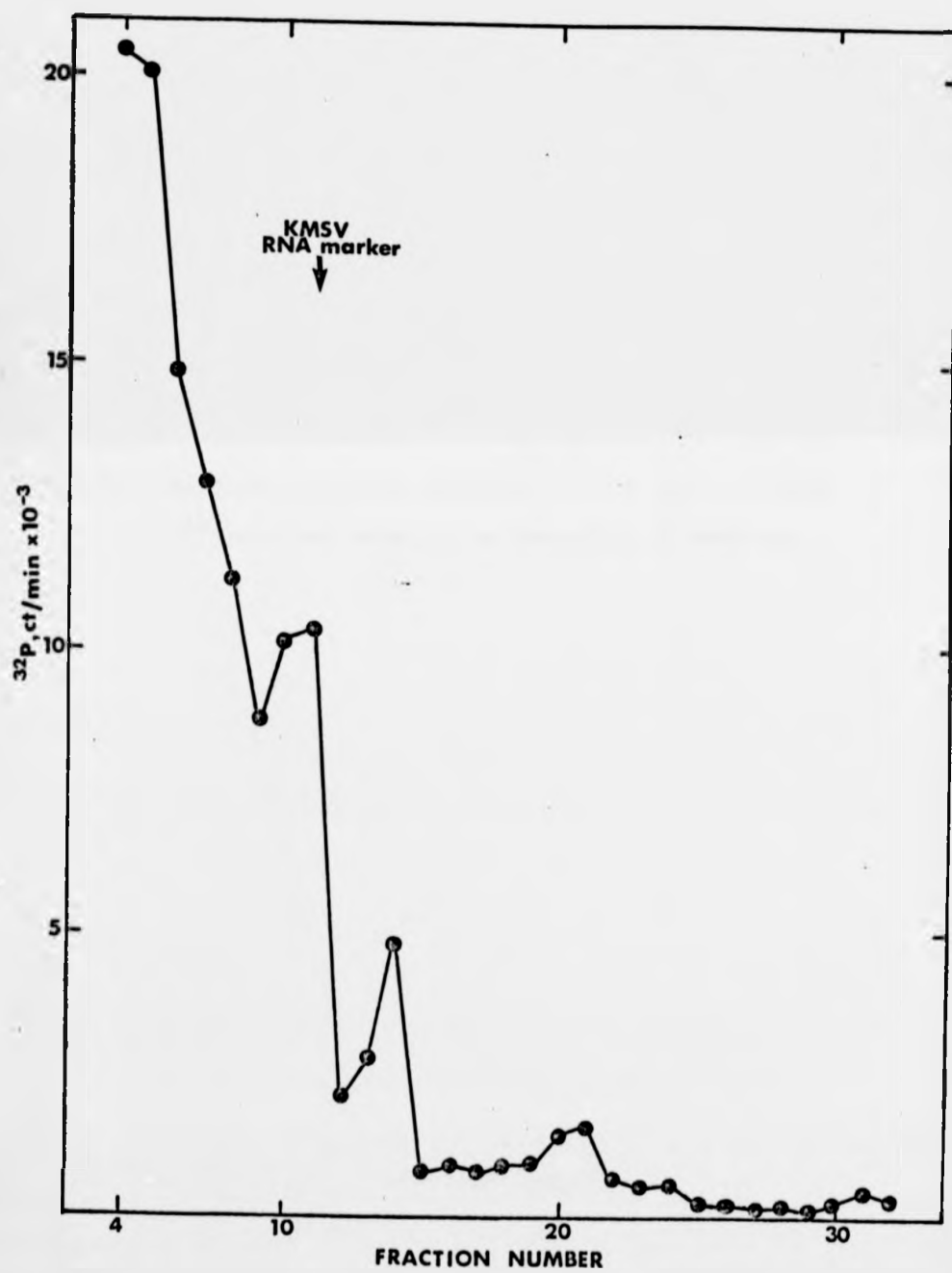


Figure 28. Velocity gradient analysis of 1.20 g/ml fraction
p³² labelled material as described in Methods.

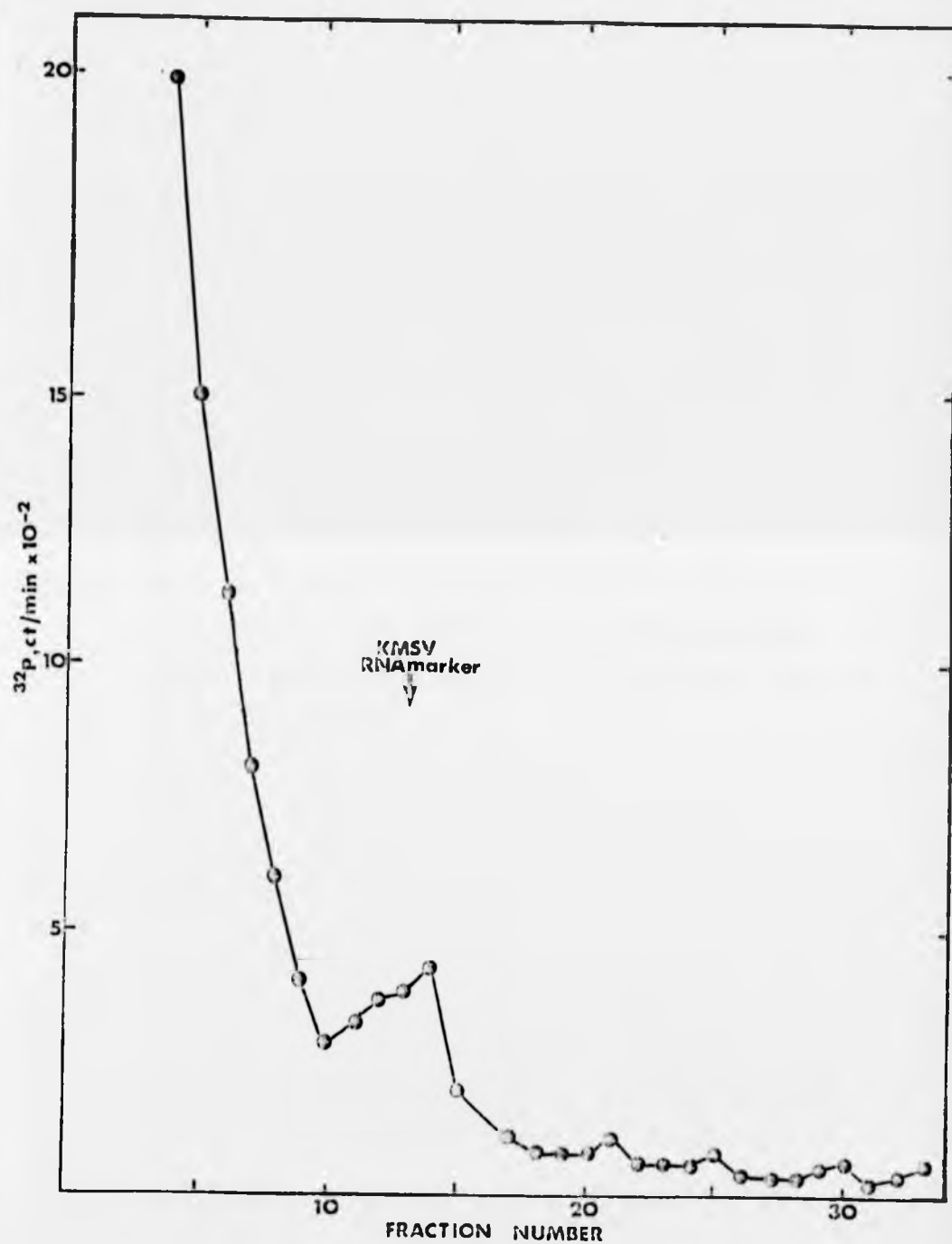


Table 11. Table of ^{32}P -counts per minute of labelled 60-70S RNA from 1.14 g/ml peak fraction from induced Drosophila pellet. Method as described in Methods; counts were made by Cerenkov counting.

TABLE 11.

FRACTION	³² P-COUNTS PER MINUTE ELUTED WITH :	
	BINDING BUFFER	ELUTION BUFFER
1	14	238
2	4949	116
3	731	34
4	331	40
5	232	36
6	310	32
7	311	
8	85	
9	76	
10	74	
11	129	
12	44	
13	39	
14	35	
15	26	

Table 12. Nuclease treatment of 60-70S moiety isolated from
1.20 g/ml peak fraction of induced Drosophila cell
fluid pellet.

RNase : pancreatic RNaseA, 1mg/1ml; RNaseT₁, 10³ units/ml,
boiled before use for 2 minutes.

DNase : used at 100 g/assay in DNase buffer; 0.01M Tris,
0.05 M NaCl, 0.02 M MgCl₂ at pH 7.5.

TABLE 12.

SAMPLE	% COUNTS TCA PRECIPITABLE
UNTREATED	51 (814 cpm)
+ RNase	22
+ DNase	63

Table 13.

Nuclease treatment of 60-70S moiety isolated from
1.14 g/ml peak fraction from induced Drosophila cell
fluid pellet. Treatment was as described in Methods.

TABLE 13.

SAMPLE	% COUNTS TCA PRECIPITABLE
UNTREATED	73
+ RNase	23
+ DNase	50

in a 30-35S species as judged by ribosomal RNA markers run on parallel gradients (Figure 29).

Figure 30 illustrates the result obtained when the same technique is applied to 1.20 g/ml peak fraction RNA.

Figure 29. Velocity gradient analysis of heat dissociated,
 P^{32} -labelled material from 1.14 g/ml fraction,
(see Methods).

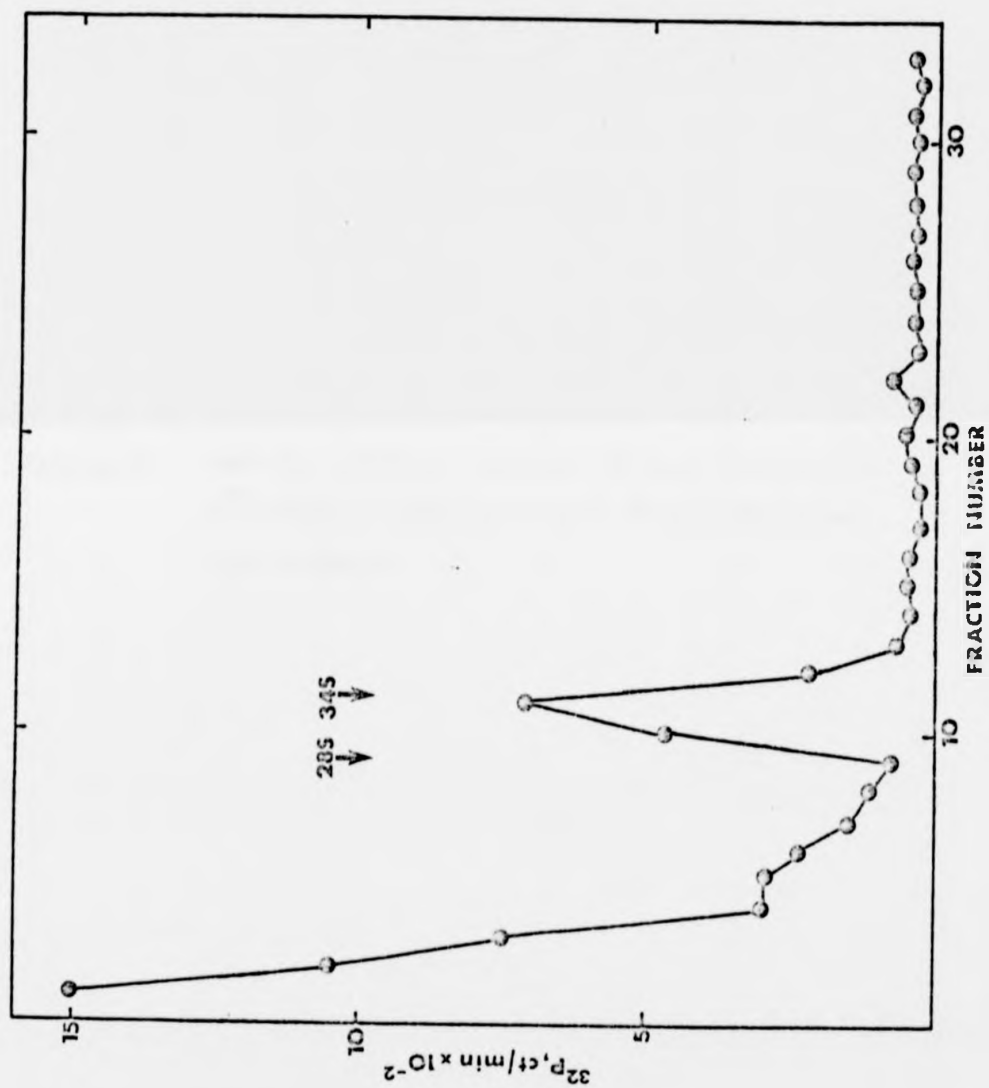
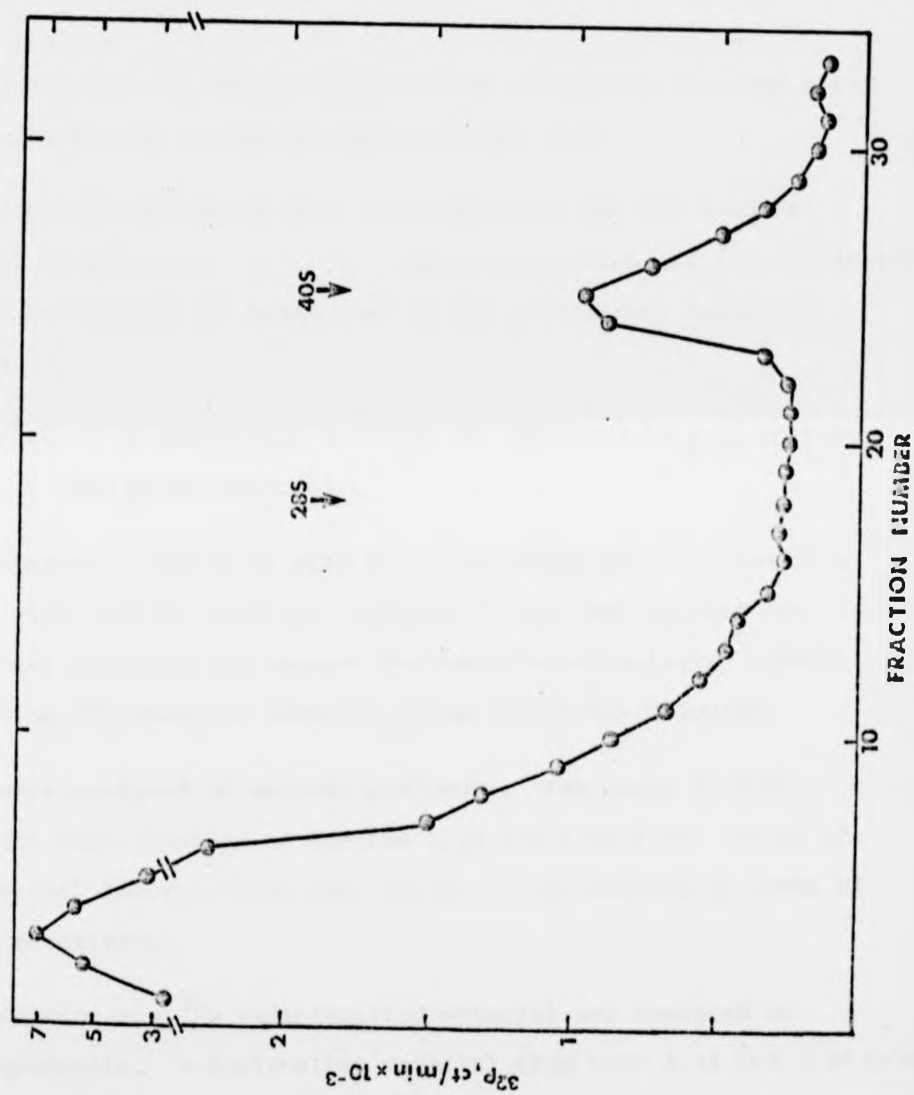


Figure 30. Velocity gradient analysis of heat dissociated,
 P^{32} -labelled material from 1.20 g/ml fraction,
(see Methods).



DISCUSSION 6.

Induction of Drosophila Cells

Induction experiments were performed in an attempt to increase yield of particles and to isolate an extracellular form.

Iododeoxyuridine proved the best inducing agent and was used in subsequent experiments. rAdT utilization and incorporation of ^3H -TTP radiolabel was again used as the preliminary detection technique.

A vast increase in polymerase activity was detected in cell culture fluid 3 - 6 days post-induction.

Further characterization of this activity, after purification of a putative viral pellet from cell culture fluids, was carried out. A polymerase activity was present in these 'viral pellets' capable of utilizing the specific template primer poly(rCm).oligo(dG).

Pellets were analysed on sucrose gradients. Two peaks of rAdT utilization were obtained of density 1.14 and 1.20 g/ml. Peaks of ^{32}P radiolabel incorporation were of equivalent density to peaks of polymerase activity.

After isolation the ^{32}P radiolabelled material was analysed on sucrose gradients. Radiolabelled material from both 1.14 and 1.20 g/ml density fractions sedimented at a position corresponding to 60-70S molecular weight RNA. RNase and DNase digestions indicated that the radiolabelled material was RNA.

1.14 g/ml 60-70S RNA was isolated from gradients and assayed for its

content of poly A sequences. Only 6% of the radiolabelled species were bound to oligo dT-cellulose columns. Therefore only a small proportion of the RNA molecules contained significant stretches of poly A sequences. It is also possible that this result is due to degradation of the RNA. It is known that the RNA of the 60-70S complex can be nicked but still hold together. On denaturation, the molecules fall apart.

1.14 g/ml and 1.20 g/ml RNA was heat denatured prior to sucrose density centrifugation. The 60-70S moiety in both cases disappeared with the subsequent appearance of label in a 30-40S species.

Again we cannot conclusively conclude that the 30-40S species observed are derived from the 60-70S complexes. The relationship of these RNA molecules from culture fluids of induced cells to the molecules isolated from uninduced cells is also unknown.

Replication of Avian and Murine Retroviruses in Insect Cells

As discussed in the Introduction, the host range of RNA tumour viruses is usually quite specific. However some, (xenotropic), viruses are capable of crossing species barriers to a certain extent, but even they cannot infect an unlimited number of cell types. This increased host range has been shown to reside in the virus coat, (see Introduction). As a corollary to our search for insect retroviruses, we were interested to determine whether insect cells could in principle support the replication of this type of virus. Therefore, experiments were carried out to determine whether RNA tumour viruses could enter insect cells.

The first type of experiment was of a biological nature. By using known RNA tumour viruses containing reverse transcriptase, we could monitor their replication within insect cells by released transcriptase activity. Virus replication should be accompanied by a concomitant increase in transcriptase activity in culture fluids. After infection of cells with virus, culture fluids were monitored for RA.dT utilizing activity. Three viruses were used in the experiments; Rous sarcoma virus (RSV) strain B77; Kirsten sarcoma virus (KMSV); and XLV (KMSV), a xenotropic virus, a pseudotype of KMSV with increased host range as described above.

Three cell lines were used; Drosophila melanogaster; Aedes albopictus^{WAR}; Chick embryo fibroblasts (CEF).

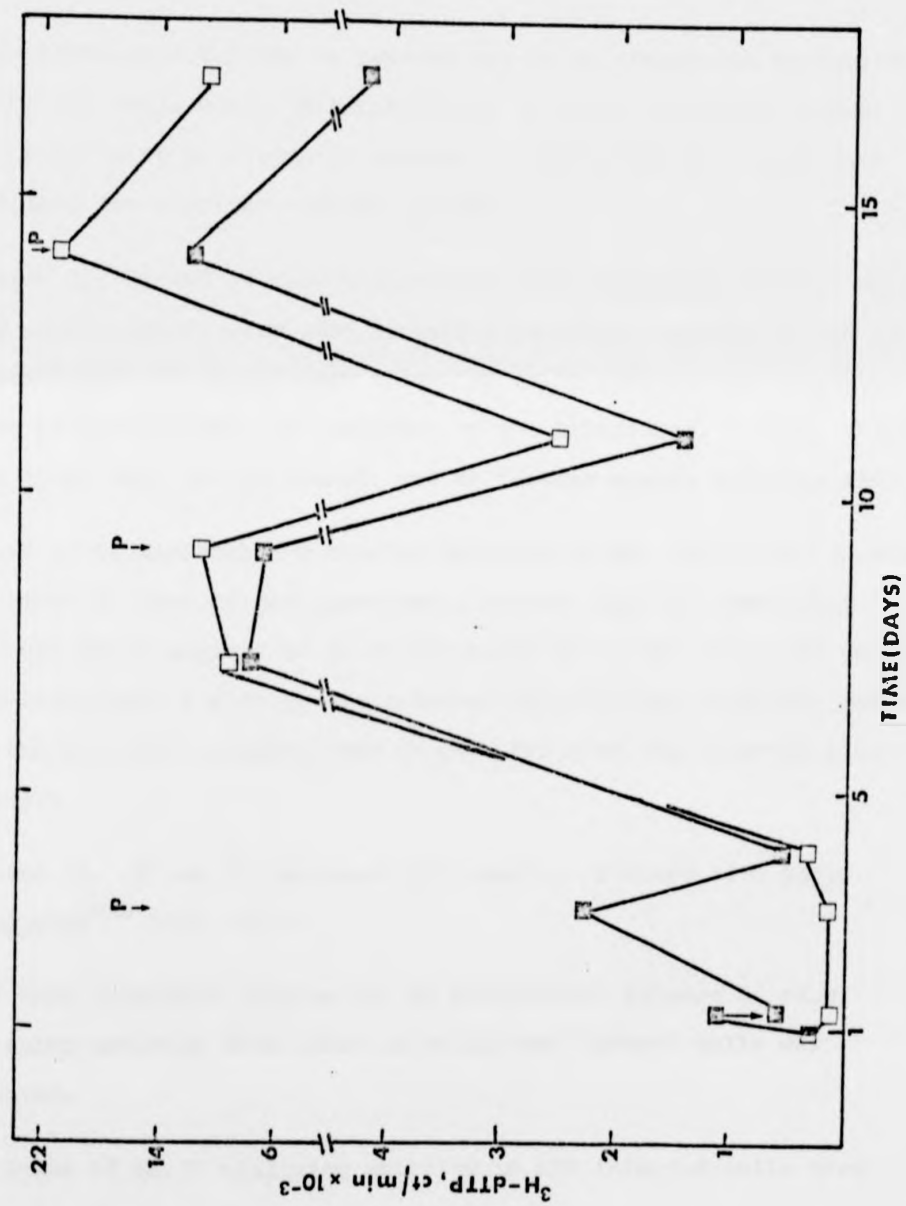
Results are shown in Figures 31 - 37. Figure 31 shows a control experiment, CEF inoculated with KMSV. As KMSV is a murine retrovirus, we would not expect to cross the species barrier with avian, CEF, cells.

Figure 31. Infection of CEF cells by KMSV : Released poly(rA).
oligo(dT) utilizing activity.

P: Passage : Cell fluids were collected pre-passage
of cells. Occasionally fluids were also
collected after passage of cells and
these results are also included where so
determined.

□: Control

■: Infected



It can be seen in Figure 31 that the rA.dT utilizing activity in both Control and Infected CEF cells is found to peak at two points. Since control cells appear to release an rA.dT utilizing activity, this result was inconclusive.

This enzyme activity may be present due to an endogenous retrovirus of the CEF cells used. Alternatively, it could represent another enzyme activity unrelated to reverse transcriptase but capable of utilizing the supplied template.primer.

Figures 32, 33 and 34 show the results with Drosophila cells. When KMSV or XLV (KMSV) were used as infecting virus, Figures 32 and 33 respectively, no significant amount of rA.dT activity was detected above background over the duration of the experiment, 3 days. After this time, cell growth ceased, and no further assays could be made.

Figure 34 illustrates the results obtained on RSV infection. During the first 10 days of the experiment, control and infected cells released small amounts of rA.dT utilizing activity. After 10 days post-infection, a rise in the released activity was detected, however the control cell activity rose in parallel with the infected cell activity.

Figures 35, 36 and 37 represent the results obtained with Aedes albopictus^{WAR} host cells.

Upon KMSV infection, Figure 35, no significant release of rA.dT utilizing activity from infected cells over control cells was detected.

Two peaks of rA.dT utilizing activity in RSV infected cells over control cells were detected, Figure 36.

Figure 32. Infection of Drosophila cells by KMSV : Released poly(rA).oligo(dT) utilizing activity.

P: Passage : Cell fluids were collected pre-passage of cells. Occasionally fluids were also collected after passage of cells and these results are also included where so determined.

□: Control

■: Infected

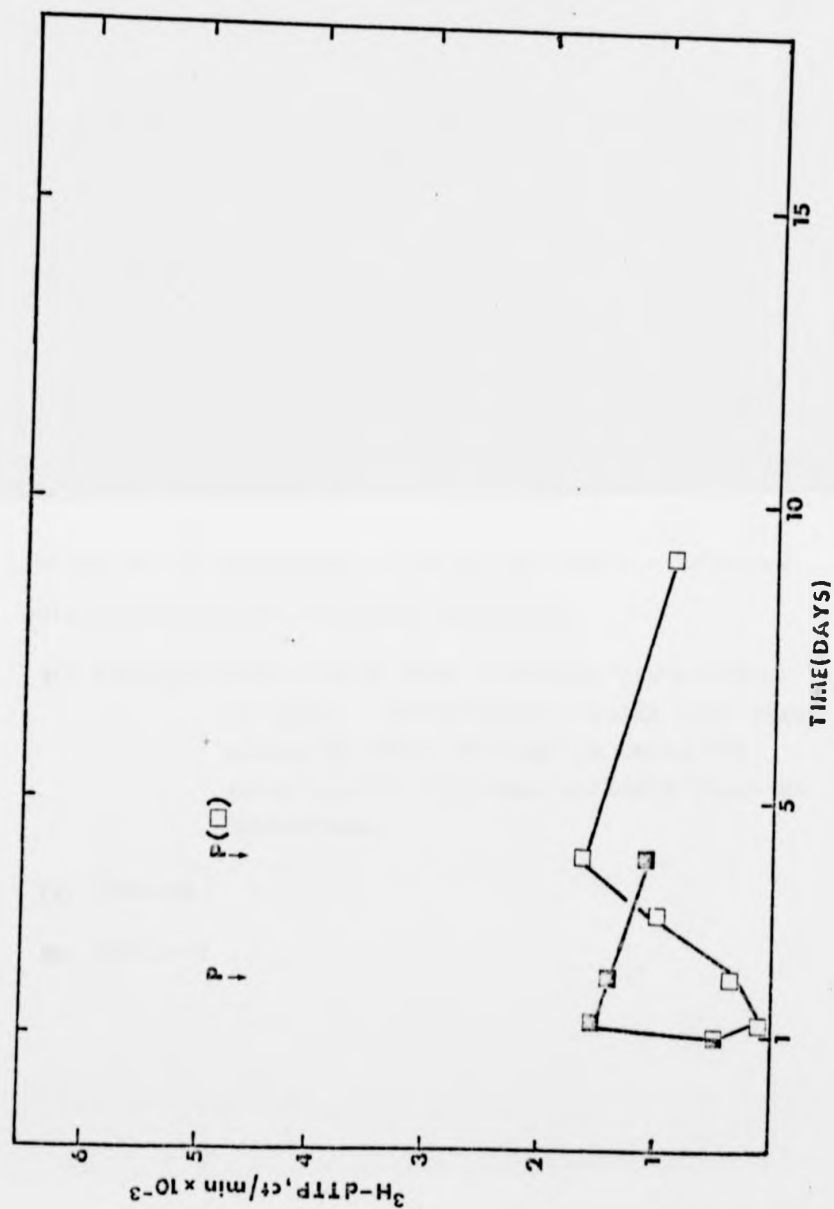


Figure 33. Infection of Drosophila cells by NLV(RMSV) : Released poly(rA).oligo(dT) utilizing activity.

P : Passage :Cell fluids were collected pre-passage of cells. Occasionally fluids were also collected after passage of cells and these results are also included where so determined.

□ : Control

■ : Infected

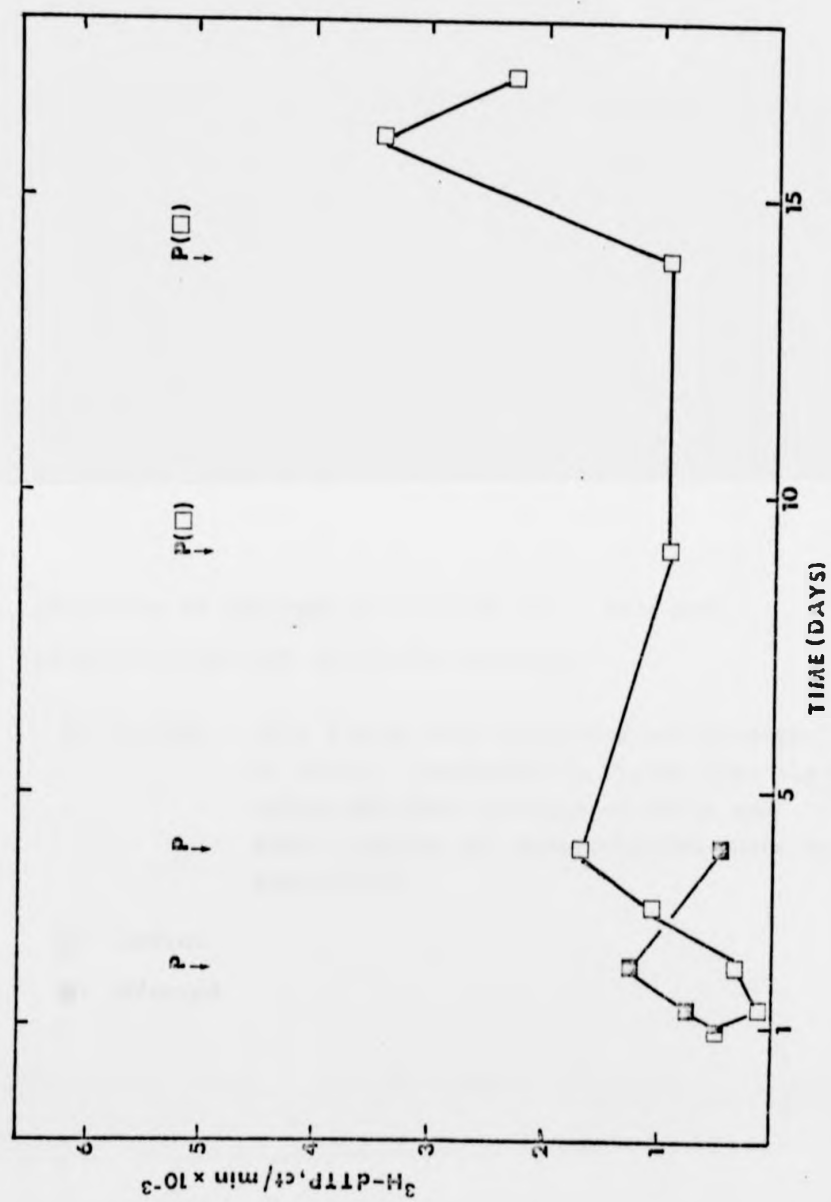


Figure 34. Infection of Drosophila cells by RSV : Released
poly(rA).oligo(dT) utilizing activity.

■: Passage : Cell fluids were collected pre-passage
of cells. Occasionally fluids were also
collected after passage of cells and
these results are also included where so
determined.

□: Control

■: Infected

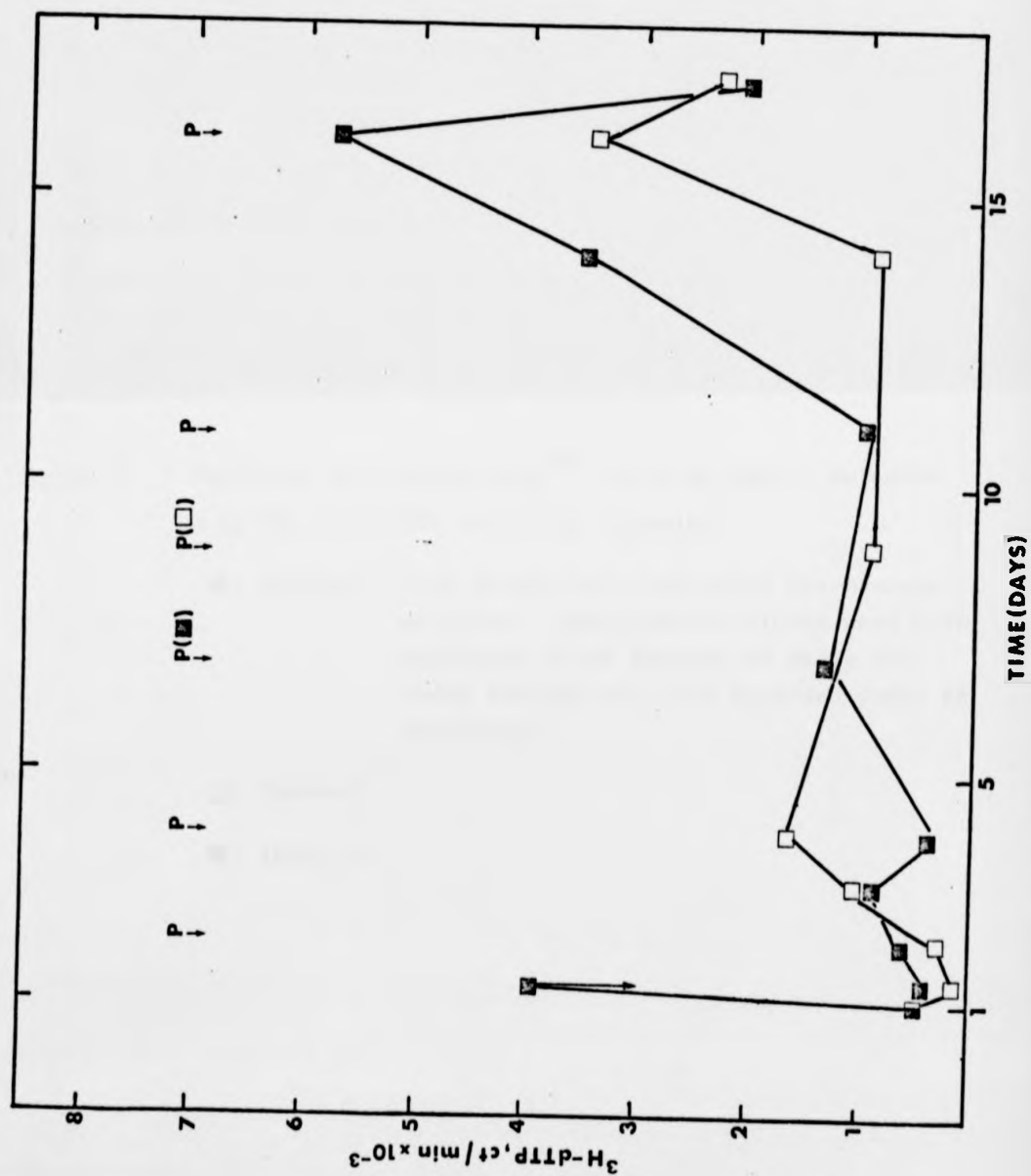


Figure 35. Infection of A.albopictus^{WAR} cells by KMSV : Released poly(rA).oligo(dT) utilizing activity.

P : Passage : Cell fluids were collected pre-passage of cells. Occasionally fluids were also collected after passage of cells and these results are also included where so determined.

□ : Control

■ : Infected

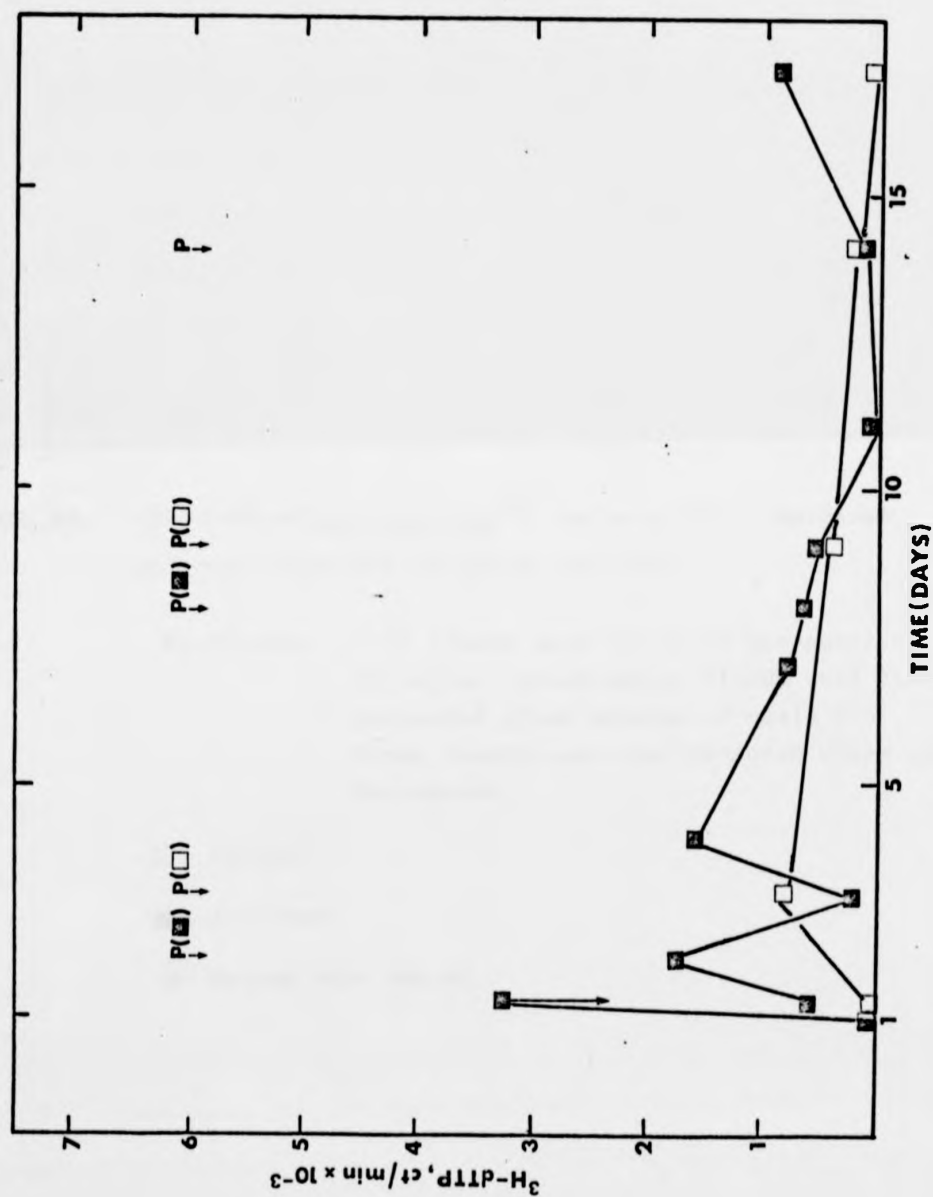


Figure 36. Infection of A.albopictus^{WAR} cells by NSV : Released poly(rA).oligo(dT) utilizing activity.

P : Passage : Cell fluids were collected pre-passage of cells. Occasionally fluids were also collected after passage of cells and these results are also included where so determined.

□ : Control

■ : Infected

I : Tested with rCm.dG

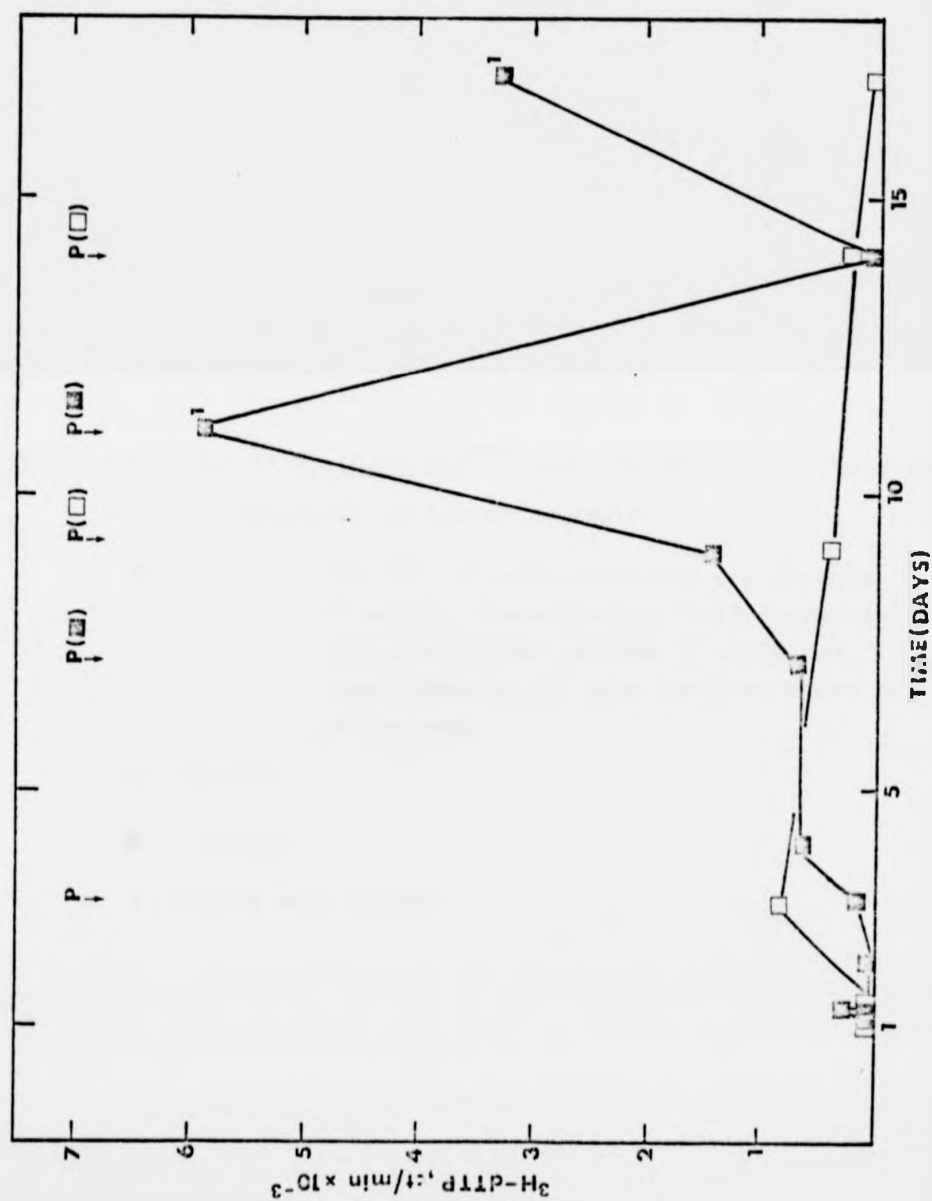


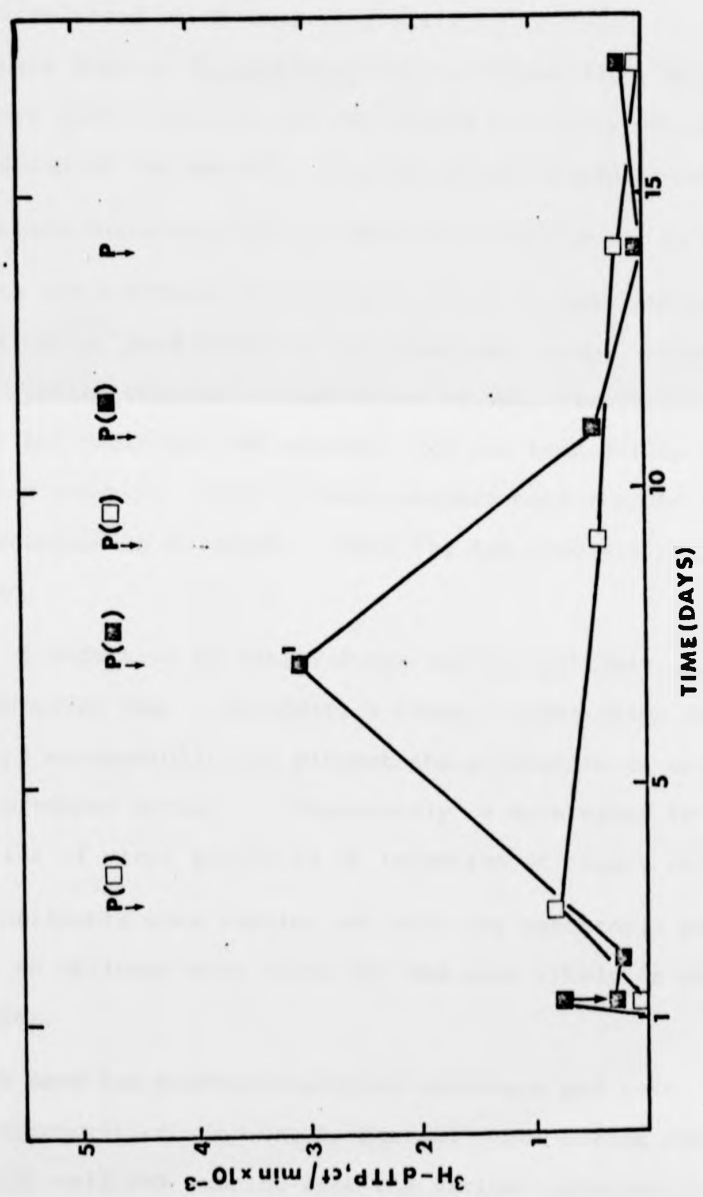
Figure 37. Infection of A.albopictus^{WAR} cells by XLV(KMSV) : Released poly(rA).oligo(dT) utilizing activity.

P: Passage : Cell fluids were collected pre-passage of cells. Occasionally fluids were also collected after passage of cells and these results are also included where so determined.

□: Control

■: Infected

1: Tested with rCm.dG



one at 10 days post-infection, one at 16 days post-infection, these peaks of activity were tested with the specific template.primers poly rCm,oligo dG. No utilization of this species was detected and these peaks of activity may represent a released cellular polymerase.

A single peak of released rA.dT utilizing activity is found in XLV (KSMV) infected cells over control A.allopietus cells, Figure 37. This peak appeared at 6 days post-infection and was tested with poly rCm,oligo dG. Again no utilization of the specific template.primers could be detected.

We can only conclude therefore that no reverse transcriptase is released.

These experiments are difficult to interpret as it is impossible to identify, specifically, production of the infecting virus. Also to be successful they require reverse transcription of RNA, transcription of DNA into genomic and messenger RNA species, and the translation of viral RNA into a protein product. This protein product must then be processed and subsequently released. That is, the complete process of viral replication.

The first stage in infection by retroviruses is the synthesis and integration of proviral DNA. In addition these viruses often infect heterologous cells successfully but without the production of progeny virus, (the non-producer state). Consequently we determined to look for the acquisition of viral sequences on infection of insect cells.

These further experiments were carried out with the xenotropic pseudotype virus since we believed this virus was the most likely to cross the species barrier.

These experiments used the Southern blotting technique and were carried out in collaboration with Dr. J. Norton. Dr. Norton was provided with total cell DNA samples from the various experimental cell lines examined. He then processed these DNA's as described below and utilized them in the Southern blot experiments. Dr. Norton produced the finished autoradiographs as shown in Figures 38, 39 and 40.

Before we give the results of these experiments we will discuss the technique and results obtained in the murine system.

Low molecular weight DNA was isolated from NIH cells infected with KMSV. This DNA was restricted to completion with the enzyme Pst I and the restriction fragments probed with DNA complementary to KMSV genomic RNA. This probe will detect specific DNA sequences with homology to the KMSV genome. Results of this restriction and probing are shown in Figure 38 Lane I.

The low molecular weight DNA fraction from the infected cells will contain the unintegrated viral DNA species. Thus the enzyme Pst I will cleave these small viral genomic sized molecules to completion. Pst I cleaves the molecules in three specific positions to produce two large internal fragments of 2.6 and 1.0 Mg d, i.e.



These fragments are clearly visible in Figure 38 Lane 1. They represent DNA sequences with specific sequence homology to the KMSV RNA genome which was used to produce the DNA probe.

The very high molecular weight species visible in Lane 1 are presumed to represent contaminating high molecular weight DNA.

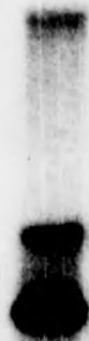
When high molecular weight DNA is isolated from CCI cells, a different pattern is observed.

CCI cells are cloned NIH cells infected with KMSV containing integrated KMSV sequences. By isolating high molecular weight DNA we are observing integrated viral sequences.

Results obtained after probing high molecular weight CCI DNA with the KMSV-specific DNA probe are shown in Figure 39 Lane 5 and Figure 40 Lane 4.

FIGURE 38. Restriction pattern obtained with Control NIH and Drosophila uninfected and XLV (KMSV) infected low molecular weight DNAs.

KMSV INFECTED NIH DNA	UNINFECTED CAT DNA pII pIII	XLV KMSV INFECTED CAT DNA pII pIII	UNINFECTED Drosophila DNA pII pIII	XLV KMSV INFECTED Drosophila DNA pII pIII
-----------------------------	-----------------------------------	---	---	--



6.0Mgd
▲
2.6Mgd
▲
1.0Mgd
▲

KMSV	UNINFECTED	XIV KMSV	UNINFECTED	XIV KMSV
INFECTED	CAT DNA	INFECTED	Drosophila	INFECTED
NIH DNA	pII pIII	CAT DNA	DNA	Drosophila DNA
		pII pIII	pII pIII	pII pIII



6.0Mgd



2.6Mgd



1.0Mgd

A large number of bands are visible, including the two fragments of 2.6 and 1.0 Mg d representing internal fragments of the KMSV DNA probe.

The other bands are non-specific and ^{can be} generated a) by host cell sequences with non-specific sequence homology to the probe (Figure 39 Lane 1) and b) by the integration of KMSV into the host cell genome. On restriction the integrated KMSV genome is specifically cleaved in the three positions shown previously to generate the two internal fragments of 2.6 and 1.0 Mg d. The host cell DNA is also cleaved. At certain positions the viral DNA sequences flanking the internal fragments will adjoin host cell sequences. These host cell sequences will be of variable size and contain sequence homology to the viral DNA sequences. However we do not pick up these sequences in our experiments since CCI DNA is a cloned cell line containing only one provirus per cell and all the extra, non-specific bands are due to hybridization to endogenous provirus, (as observed in Figure 39, Lane 5 and Figure 40, Lane 4).

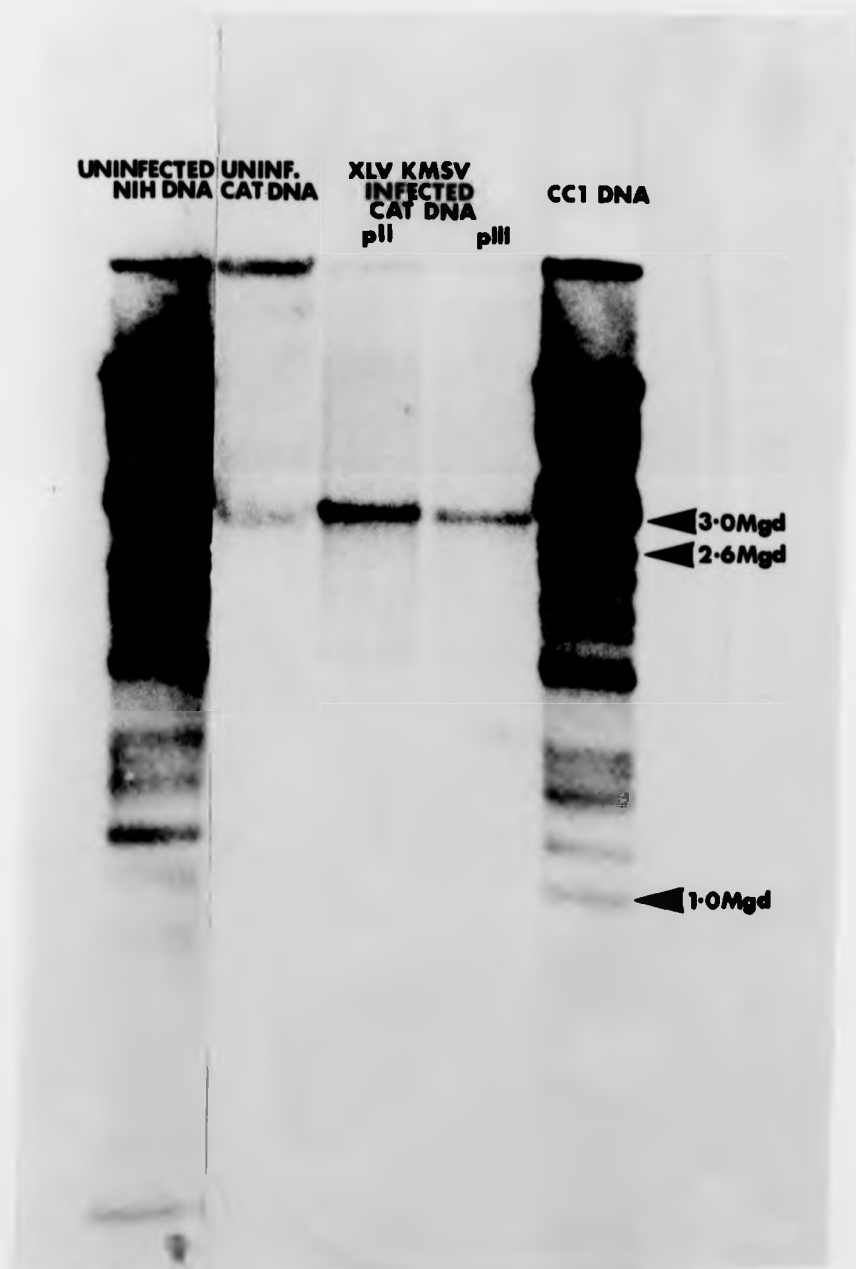
Thus we can distinguish those bands of 2.6 and 1.0 Mg d which are specific to the KMSV genome from those non-specific bands which arise from the integration of the viral genome into the host cell DNA.

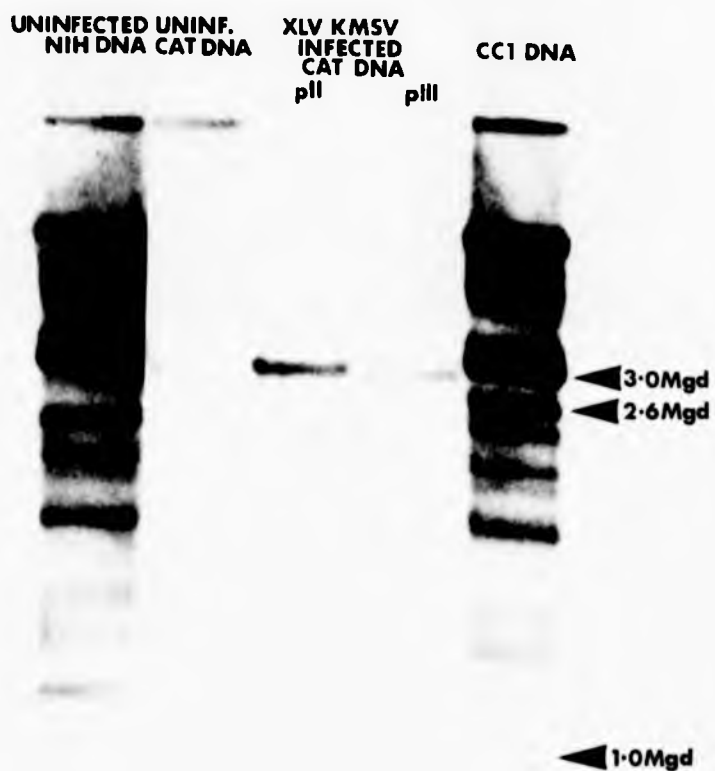
By this technique we obtained a method of detecting integrated and unintegrated viral-specific sequences. By using XLV(KMSV) in these experiments we could employ the same probe as in the murine control experiments, since the KMSV genome is present in the pseudotype virus.

Drosophila melanogaster and Canine thymus (CAT) cells, as a mammalian control line, were utilized in this experiment.

Cells were infected with XLV(KMSV) and both high and low molecular weight DNAs were isolated from infected and control cells at passage numbers two and three.

FIGURE 39. Restriction pattern obtained with Control and CAT uninfected and XLV (KMSV) infected high molecular weight DNAs.





CAT cells are known to have no KMSV related sequences (Norton and Avery, unpublished data).

Figure 38 illustrates the results of probing low molecular weight DNA isolated from Drosophila and CAT cells.

It is possible that the XLV(KMSV) genome could enter the insect cell but may not become integrated. We were interested to see if any such unintegrated sequences existed within the low molecular weight DNA from infected cells.

Lane 1 is a control lane showing KMSV infected NIH cell DNA as described above. The specific bands of 2.6 and 1.0 Mg d are clearly visible.

Lanes 2 and 3 represent uninfected CAT DNA pII and pIII. No hybridization is observed, thus no viral specific species are present in this low molecular weight DNA.

Lanes 4 and 5 are the restriction patterns obtained with XLV(KMSV) infected CAT low molecular weight DNA, passage two and three respectively.

A single restriction fragment can be seen in Lane 4 of 6×10^6 d m.w. This band is clearly not a KMSV internal fragment, however it does share sequence homology with the KMSV cDNA probe. The origin of this species is unknown.

Lanes 6 and 7 contain low molecular weight uninfected Drosophila DNA pII and pIII. Lanes 8 and 9 contain XLV(KMSV) infected low molecular weight Drosophila DNA pII and pIII. No hybridization is observed with the cDNA KMSV probe in any of these lanes. Thus no unintegrated KMSV-sequences related moiety could be detected within the invertebrate cell line. This may be a reflection of the quantity or

quality of XLV(KMSV) infection in invertebrate cells.

Figure 39 shows the results obtained with high molecular weight DNA from infected and uninfected CAT cells.

Lane 1 illustrates the fragmentation pattern observed with uninfected control NIH DNA. No ^{specific} hybridization to the KMSV-specific probe is seen, however numerous non-specific fragments are observed.

Lane 5 shows the pattern obtained with CCI DNA. As described earlier many bands are present, however the two specific fragments of 2.6 and 1.0 Mg d are clearly visible.

A small amount of background hybridization is present in Lane 2 containing uninfected CAT DNA. However no virus-specific fragments of 2.6 or 1.0 Mega daltons are visible.

Lanes 3 and 4 represent the patterns obtained with XLV(KMSV) infected CAT DNA from the second and third passage post-infection, (see Methods).

An additional fragment of 3.0 Mg d is observed. This does not correspond to the Pst I fragments of KMSV DNA and as such is an unknown provirus with some sequence homology to the KMSV cDNA probe. We assume this represents integration of the (XLV)XLV component of the infecting virus. This is likely since (i) size is that expected for a double-stranded virus genome, and (ii) the band shows homology to the probe. This is expected with XLV.

Figure 40 illustrates the restriction patterns obtained from high molecular weight DNA isolated from Drosophila cells.

Lane 4 shows the restriction pattern of control CCI DNA showing the specific 2.6 and 1.0 Mg d fragments and numerous non-specific fragments, described earlier.

quality of XLV(KMSV) infection in invertebrate cells.

Figure 39 shows the results obtained with high molecular weight DNA from infected and uninfected CAT cells.

Lane 1 illustrates the fragmentation pattern observed with uninfected control NIH DNA. No ^{specific} hybridization to the KMSV-specific probe is seen, however numerous non-specific fragments are observed.

Lane 5 shows the pattern obtained with CCI DNA. As described earlier many bands are present, however the two specific fragments of 2.6 and 1.0 Mg d are clearly visible.

A small amount of background hybridization is present in Lane 2 containing uninfected CAT DNA. However no virus-specific fragments of 2.6 or 1.0 Mega daltons are visible.

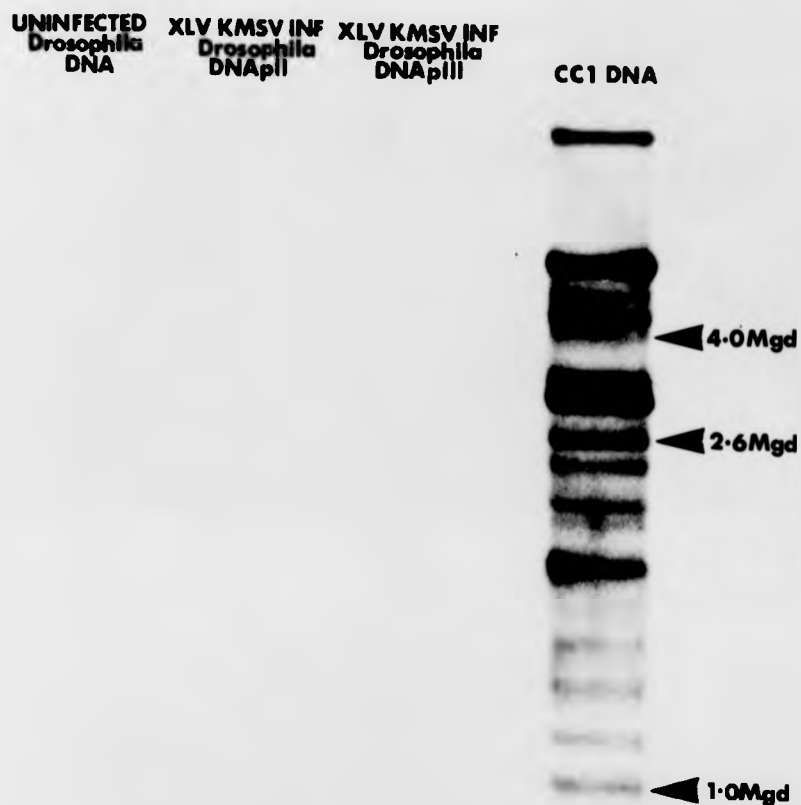
Lanes 3 and 4 represent the patterns obtained with XLV(KMSV) infected CAT DNA from the second and third passage post-infection, (see Methods).

An additional fragment of 3.0 Mg d is observed. This does not correspond to the Pst I fragments of KMSV DNA and as such is an unknown provirus with some sequence homology to the KMSV cDNA probe. We assume this represents integration of the (XLV)XLV component of the infecting virus. This is likely since (i) size is that expected for a double-stranded virus genome, and (ii) the band shows homology to the probe. This is expected with XLV.

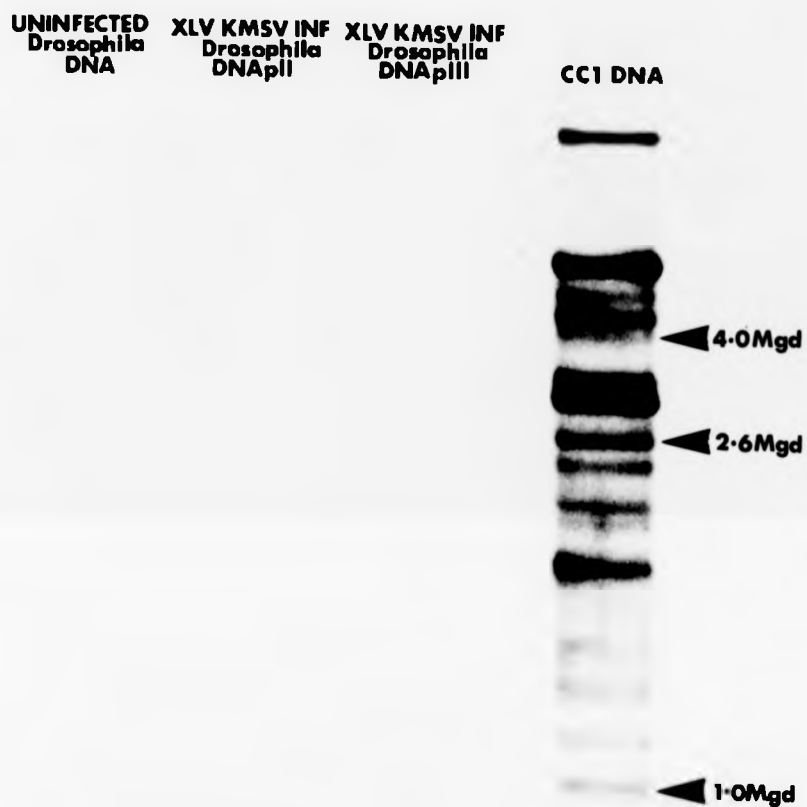
Figure 40 illustrates the restriction patterns obtained from high molecular weight DNA isolated from Drosophila cells.

Lane 4 shows the restriction pattern of control CCI DNA showing the specific 2.6 and 1.0 Mg d fragments and numerous non-specific fragments, described earlier.

FIGURE 40. Restriction pattern obtained with Controls and Drosophila uninfected and XLV (KMSV) infected high molecular weight DNAs.



Although clearly visible on the autoradiograph the 4.0 Mg d band does not appear on the photograph.



Although clearly visible on the autoradiograph the 4.0 Mg d band does not appear on the photograph.

Lane 1 contained restricted uninfected Drosophila DNA. No hybridization with the KMSV specific probe was observed. Thus no KMSV specific sequences were present in the uninfected high molecular weight Drosophila DNA.

Lanes 2 and 3 are the restriction fragments from XLV(KMSV) infected Drosophila cell DNA, passage two and three respectively.

An additional fragment of 4.0 Mg d was present on the autoradiograph, however due to the lack of intensity of this band it does not appear on the photograph.

The 4.0 Mg d fragment does not correspond in size to any of the Pst I fragments of KMSV DNA nor to any fragment seen in XLV(KMSV) infected CAT cells.

From this result it can be stated that a new set of sequences has entered the XLV(KMSV) infected Drosophila DNA, with some sequence homology to KMSV cDNA. The origin and nature of these sequences cannot be deduced from these results.

DISCUSSION 7.

Replication of Avian and Murine Retroviruses in Insect Cells

On infection of the insect cells with known retroviruses, no conclusive results were obtained using polymerase activity as a measure of replication.

With Drosophila cells no significant increase in rAdT utilizing polymerase levels in extracellular fluids of cells infected with KMSV, RSV or XLV was detected over uninfected cells.

When Aedes albopictus^{WAR} cells were used no significant result was obtained when KMSV was the infecting virus. With XLV one peak of polymerase activity was detected in infected cells, with RSV two peaks were obtained. These peaks of rAdT utilizing activity were tested with the specific template.primer poly(rCm).oligo(dG). However no activity was detected with this template.primer.

By using this method of detection we cannot determine whether or not virus particles have infected the host cell. We are assaying for reverse transcriptase, therefore virus particles must infect host cells, be transcribed and translated to form the enzyme. Enzyme must then be processed into its active form and released from the cells, (see Results section). Any of these stages in enzyme formation could be blocked in invertebrate cells so that no enzyme or an inactive form is produced.

Therefore we performed Southern blot experiments using XLV (KMSV) as infecting virus, (see Methods).

When low molecular weight DNA from uninfected CAT cells is probed

with KMSV specific DNA no hybridization is observed. (Figure 38). Restriction of control KMSV infected NIH DNA produces two internal fragments of the KMSV genome of 2.6 and 1.0 Mg d, (Figure 38, lane 1). No probing low molecular weight DNA from XLV(KMSV) infected CAT cells a single species of 6×10^6 d is observed, (Figure 38, lane 4). Since this species is not present in control KMSV infected NIH DNA it does not represent KMSV DNA but contains sequence homology with this DNA. This sequence probably derives from the XLV(MLV) component of the virus stock, (see later). No hybridization was observed in the low molecular weight DNA samples from infected or uninfected Drosophila cells. Thus no unintegrated viral related sequences are present in the Drosophila DNA samples.

Figure 39 represents a control experiment using high molecular weight DNAs. Any hybridization observed should be due to integrated viral sequences, or host cell sequences with sequence homology to the KMSV specific probe.

Lane 5 of Figure 39 contains CCI DNA restriction fragments. CCI cells contain integrated KMSV. Thus the restriction fragments contained in Lane 5 show the two internal fragments of KMSV, generated by the Pst I restriction, of 2.6 and 1.0 Mg d. Also shown are numerous non-specific bands with sequence homology to the KMSV probe. Non-specific bands are also visible in the control uninfected NIH DNA sample (Figure 39 Lane 1) however no KMSV specific bands can be observed.

In XLV (KMSV) infected CAT DNA (Figure 39 lanes 3 and 4) a single additional fragment of 3.0 Mg d is observed.

Data collected by Dr. J. Norton, (personal communication), indicates that this 3.0 Mg d fragment is an internal fragment of an unidentified

provirus previously seen at numerous sites in the KCAT genome ie. CAT cells infected with XLV(KMSV) on a previous occasion.

As indicated earlier this fragment is not observed in uninfected CAT cells nor in CCI DNA. This band probably represents the XLV(XLV) component of the XLV(KMSV) virus stock. As stated earlier this is likely since a) its size is that expected for a double-stranded virus genome and b) the band shows homology to the probe - to be expected with XLV.

It is probable that the 6.0 Mg d species seen in the low molecular weight, XLV(KMSV) infected CAT DNA, (Figure 38), represents the unintegrated form of this unknown provirus.

In uninfected Drosophila high molecular weight DNA no hybridization to the KMSV specific probe is observed, (Figure 40, lane 1). However in XLV(KMSV) infected Drosophila DNA a 4.0 Mg d species is present. (Unfortunately due to the lack of intensity this band does not appear on the photograph).

This moiety does not correspond to any of the fragments identified in XLV(KMSV) infected CAT cells nor to any specific KMSV DNA fragments.

The origin of this species is unknown, however sequences with homology to KMSV DNA sequences have been acquired by the Drosophila genome after the addition of XLV(KMSV) to their surface. This result implies that Drosophila cells are not completely resistant to retro-viral infection.

Infection of Mammalian and Invertebrate Cells with Induced Virus Fluids

In order to classify a biological agent as a virus, it is necessary to demonstrate the infectivity of that agent.

Culture fluids were taken from Drosophila cells induced with IDUR and diluted ten-fold in order to reduce the rAdT utilizing activity present. This was done in order to detect any significant increase in rAdT utilizing activity in infected cell culture fluids.

Five cell lines were treated with DEAE dextran (see Methods) and infected with induced Drosophila fluids. Cell lines used were NRK, NIH 3T3, 1855.B4, Aedes albopictus^{WAR} and Drosophila melanogaster. Infected and mock-infected cell culture fluids were assayed for rAdT utilizing activity and results are shown in Figures 41, 42, 43, 44 and 45.

Figure 41 illustrates the results obtained with NIH 3T3 cells. No significant increase in rAdT utilizing activity in infected culture fluids was detected over mock-infected fluids.

This result was also found when NRK cells were used (Figure 42).

Figure 43 shows results with 1855.B4 cells. These cells contain rescuable Harvey sarcoma virus and a series of focus assays were also carried out on the cell culture fluids (see earlier). If the induced particle can infect these cells the Harvey Sarcoma Virus may be rescued thus releasing rAdT utilizing and focus forming activities.

A significant increase in rAdT utilizing activity in infected cell culture fluids over mock-infected culture fluids was detected eight days post-infection. However when cell culture fluids were assayed

Figure 41. Addition of induced Drosophila cell fluids to NIH 3T3
cells : Released poly(rA).oligo(dT) utilizing activity.

pp : Post passage

prp : Pre passage

Δ : Control

▲ : Infected

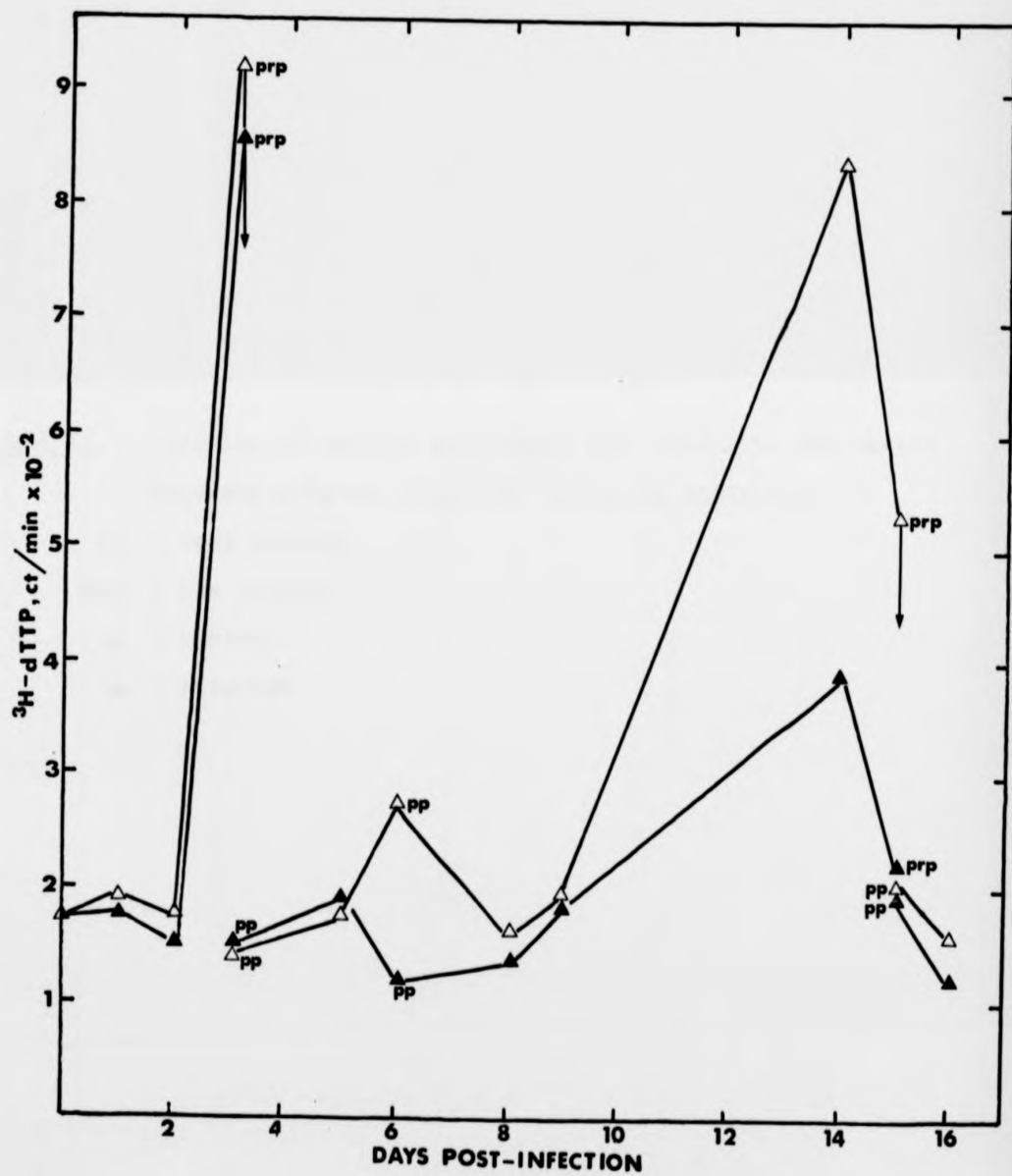


Figure 42. Addition of induced Drosophila cell fluids to NRK cells:
Released poly (rA).oligo (dT) utilizing activity.

pp : Post passage

prp : Pre passage

Δ : Control

▲ : Infected

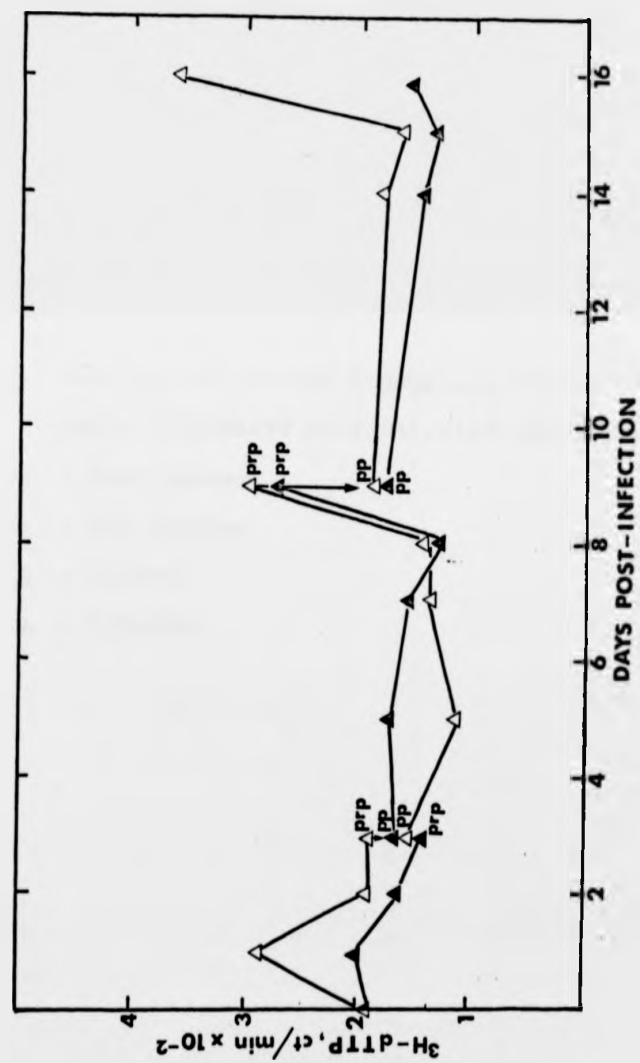


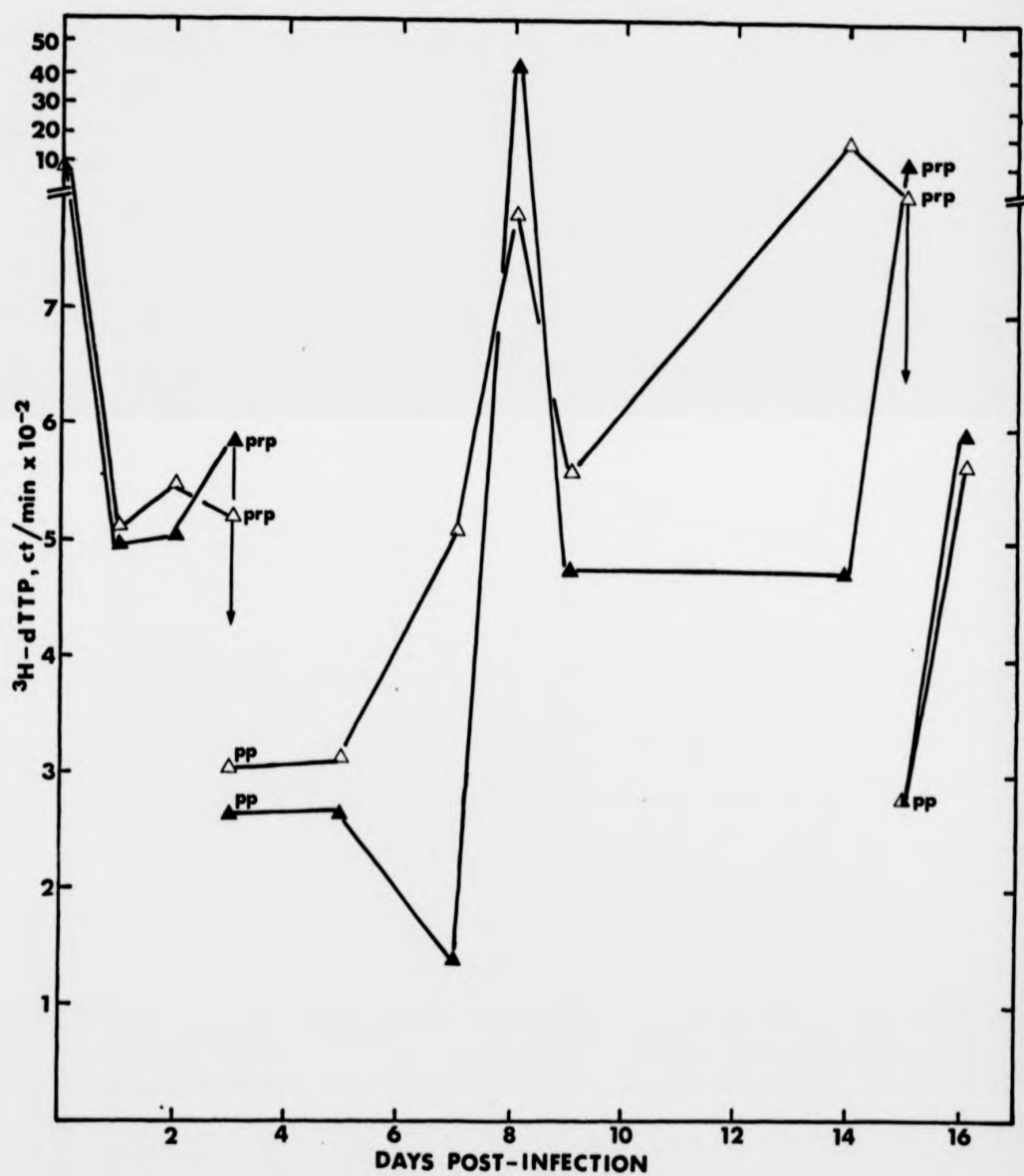
Figure 43. Addition of induced Drosophila cell fluids to 1855.B4
cells : Released poly(rA).oligo(dT) utilizing activity.

pp : Post passage

prp : Pre passage

Δ : Control

▲ : Infected



for focus forming activity, no foci were detected.

In Figure 44 results obtained with Aedes albopictus^{WAR} are shown. As with NIH 3T3 and NRK cell lines, no increase in rAdT utilization in infected cell culture fluids was detected. At all sample times, mock-infected cell culture fluids incorporated more ³H-TTP using rAdT as template.primer than infected cell culture fluids.

When Drosophila melanogaster cells were infected (Figure 45), only one sample, 15 days post-infection, incorporated more ³H-TTP, using rAdT as template.primer, than mock-infected cultures. This level of activity, however, decreased post passage to a level below that of mock-infected cells.

Figure 44. Addition of induced Drosophila cell fluids to
A.albopictus^{WAR} cells : Released poly(rA).oligo(dT)
utilizing activity.

prp : Pre passage

Δ : Control

▲ : Infected

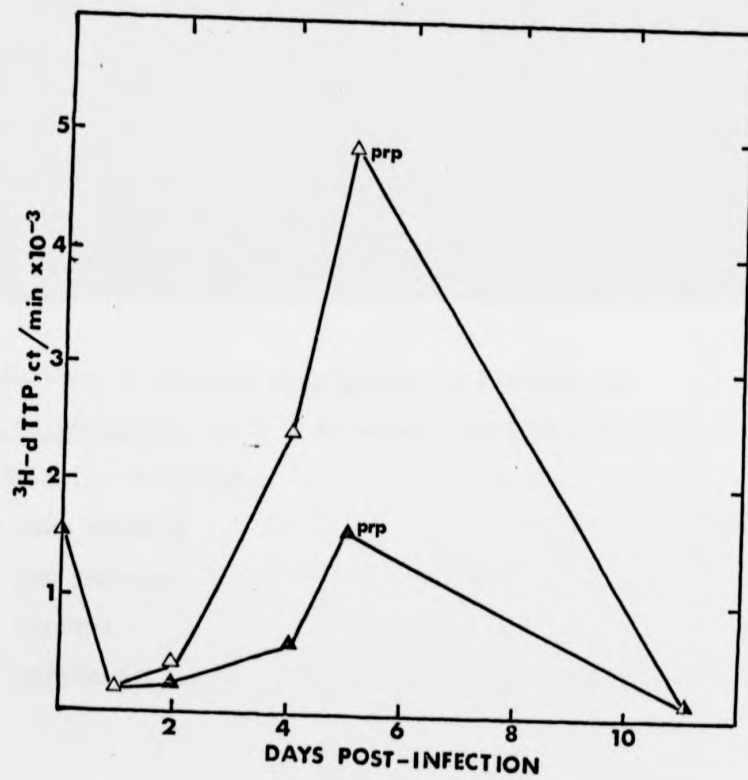


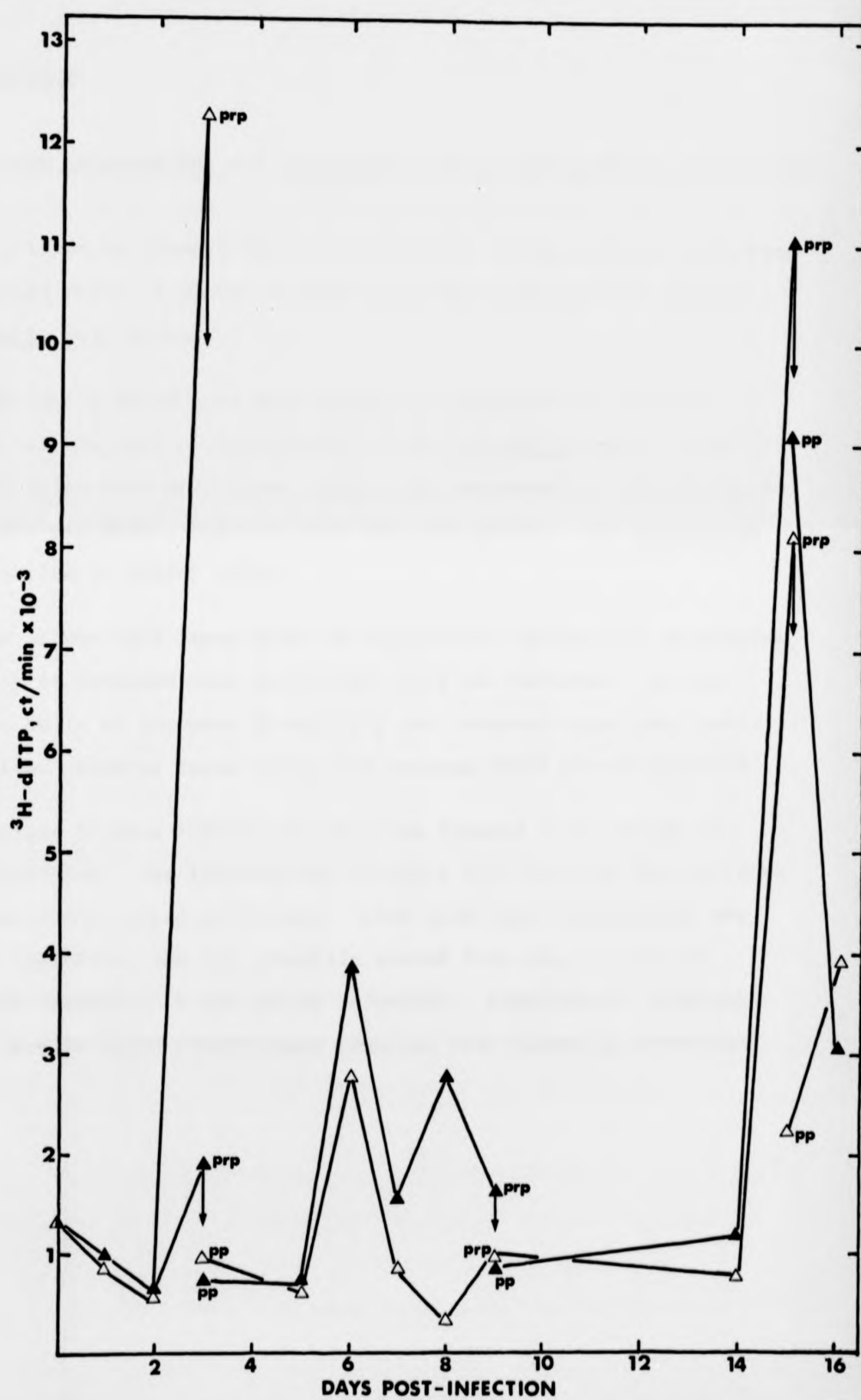
Figure 45. Addition of induced Drosophila cell fluids to
D.melanogaster cells : Released poly(rA).oligo(dT)
utilizing activity.

pp : Post passage

prp : Pre passage

Δ : Control

▲ : Infected



DISCUSSION 8.

Infection of Mammalian and Invertebrate Cells with Induced Viral Fluids

In an attempt to demonstrate the infectivity of the induced agent from Drosophila cells, a number of cell lines were treated with induced Drosophila cell fluids.

rAdT utilizing activities were assayed in infected cell culture fluids as a measure of replication of the Drosophila agent. When 1855.B4 cells were used focus assays were performed on cell fluids to assay for any HaMSV 'rescued' from the cell genome - the Drosophila agent acting as helper virus.

In four of the cell lines used, no significant increase in polymerase activity in infected over uninfected cells was detected. In the 1855.B4 cells an increase in activity was detected eight days post-infection. However focus assays for rescued HaMSV proved negative.

The failure to show infectivity with the induced viral fluids is not surprising. The invertebrate particle must overcome the barriers to infection outlined previously. Also wild type retroviruses are poorly infections and are generally passed from cell to cell by vertical transmission and not by infection. Experimental infection of cells with known retroviruses requires high titres of infecting virus.

DISCUSSION

A-, B-, C- and D- type retrovirus particles have been isolated from the cells of many species of vertebrate, as described in the Introduction. Conversely, these viruses have not been reported in invertebrates, although many virus groups are associated with invertebrates, (see earlier).

We were interested to determine whether invertebrates could harbour retroviruses for two main reasons.

Firstly, invertebrates could potentially act as a reservoir and also as vector for these viruses. If detected this would be the first report of such viruses in invertebrate cells and thus extend the known host-range of the retroviruses.

Secondly, if present in invertebrates, retroviruses could be contaminants of preparations of other viruses disseminated for use as viral pesticides.

Initial screening of insect cells for the presence of Retroviruses was carried out in three ways: Focus and Plaque assays and Reverse Transcriptase assays.

Focus and Plaque assays both proved negative. These assays are extremely limited in their range of detection. The first barrier to a result is the temperature difference in the two systems being used. This may be sufficient to prevent detection of any particles present. Another barrier is that of the cell surface. It has already been discussed as a major factor in determining resistance/susceptibility to infection. In order to exhaust the possibility of biological detection more cell types need to be used in the assays,

including invertebrate cells of various types.

The lack of a result for this experiment does not indicate the total absence of Retrovirus particles from these cells. It does indicate our inability to detect any such viruses.

The 'Reverse Transcriptase' assay was a useful tool for rapid preliminary screening. Using rAdT as template.primer a number of polymerases could incorporate ^3H -TTP into this template.primer. However rAdT is relatively non-specific i.e. many polymerases, other than reverse transcriptase, can utilize this template.primer. Thus for positive identification of reverse transcriptase the more specific poly(rCm).oligo(dG) was utilized. This chemical was very expensive and therefore was applied only to putative positive samples from the rAdT screening.

Fractionation of rAdT positive cell lines was crude and much of the polymerase activity may have been lost by the freeze/thaw treatment used to disrupt the cells. Other methods of fractionation were employed, eg. sonication and Dounce fractionation, but in both cases polymerase activity was reduced below that obtained by the freeze/thaw technique (Data not shown).

The Drosophila melanogaster cell line showed the highest response to poly(rCm).oligo(dG) and was subjected to further investigation.

Utilization of the specific .template indicated the presence of a true reverse transcriptase or a previously undefined enzyme capable of utilizing this molecule.

When analysing the exogenous reaction of the Drosophila polymerase activity it does not show the typical template.primer utilization ratios shown by other reverse transcriptases. However, the order

of preferential utilization of template primers is identical to that of known reverse transcriptases.

Other characteristics of the Drosophila melanogaster polymerase, temperature dependence and divalent cation requirements were similar to those expected for reverse transcriptase. However these characteristics, and the template primer utilization could have been better determined if a more purified enzyme preparation was available. Given a sufficient quantity of cells, the preparation of a pure polymerase devoid of subcellular components may be possible using conventional techniques.

Problems may be encountered in the physical "bulking-up" of cells. Quantity of cells may be overcome by adapting the cell line to grow on glass roller-bottles, thus increasing yield by several-fold. Indeed it is possible to grow the Drosophila cell line on glass, however several experiments must be carried out to determine if this cell population reacts in the same way as those cells grown on plastic.

Despite the problem of quantity, the preliminary results from the exogenous reactions of the Drosophila polymerase indicated characteristics similar to those of reverse transcriptases.

Data from the endogenous reaction indicates an RNA template is associated with the Drosophila polymerase activity. This reaction is dependent upon the endogenous template of the polymerase activity rather than the enzymes ability to utilize an added template primer. However owing to the uncertain purity of the polymerase and the fragility of RNA the results of this experiment may be grossly affected.

Further characterization of the reaction products could have been carried out, eg. size and sequence. A further test of product origin would have been to hybridize the DNA products back to the RNA template. This was not done due to a lack of material. Also we would expect the products to hybridize back to the Drosophila sample since it was copied from it. It is the nature of the sample which requires further purification and characterization.

The polymerase appears to be antigenically distinct from AMV reverse transcriptase by antibody analysis. Further analysis, eg. with anti-Primate or anti-Feline reverse transcriptases, could prove useful. Since the Drosophila polymerase has been isolated from the invertebrate system it may prove that the enzyme is antigenically distinct from all vertebrate reverse transcriptases. However if the Drosophila polymerase is the ancestral reverse transcriptase some antigenic similarity should be observed.

A limited amount of RNase H activity was detected within the Drosophila polymerase fractions. A series of modifications to reaction mix and conditions of incubation, or by further polymerase purification, may have increased this activity.

Electron microscopy of the Drosophila cell line did not reveal any B- or C- type particles but toroidal forms were observed similar to intracytoplasmic A-type particles though of smaller diameter. This type of electron microscopy is a matter of interpretation. A more specific type of electron microscopic work eg. immuno electromicroscopy could be employed if antibody could be raised against the Drosophila particle. However this technique requires a specific, purified, antibody preparation and therefore the original sample should be characterized.

An analysis of ³H-uridine labelled disrupted Drosophila cell material revealed a peak of radioactivity possessing a buoyant density coincident with that of the Drosophila polymerase activity. This labelled material possessed poly(A) sequences and was susceptible to alkali and RNase treatment, characteristics of RNA.

Characterization of this RNA on sucrose velocity gradients revealed a peak of radioactivity sedimenting slightly ahead of KMSV 60-70S RNA included as a marker.

It proved impossible to heat denature this RNA to observe the appearance of 30-35S RNA, a characteristic of retroviral RNA. However when RNA preparations were heat denatured prior to gradient centrifugation a broad peak of radioactivity at 30-35S was observed with the disappearance of the 60-70S RNA. Our inability to heat denature the 60-70S RNA from sucrose gradients was probably due to impurities contaminating our sample post-purification. Thus, although it is likely, we cannot conclude that the 30-35S species is derived from the 60-70S RNA moiety.

A more incisive method of analysing these RNA species would be to utilize agarose gel electrophoresis. Size and complexity of the RNA's could then be established.

It can be argued that the Drosophila melanogaster cell line was contaminated by an exogenous retrovirus early in this work. However the initial work was performed in the Unit of Invertebrate Virology in Oxford where retroviruses are not used. Dr. P. Scotti, who provided the cell line, has stated that no viruses were present in the line when presented to the Unit. Also Drosophila cells were routinely tested for the presence of mycoplasma and were found to be negative.

In addition the characteristics of the Drosophila particle are also sufficiently different from avian and murine retroviruses, used in Warwick, to rule out contamination by these.

Although the intracellular Drosophila particle was of great interest it was decided to attempt the induction of the Drosophila cells for the following reasons:

- a) Levels of polymerase available for characterization could have been increased by inducing invertebrate cells.
- b) In order to demonstrate infectivity an extracellular form is necessary, which leads to
- c) An extracellular form could be employed to increase levels of virus sample.

After induction of Drosophila cells a rise in extracellular rAdT utilizing activity was detected after 3-6 days. Purification of a 'viral pellet' from these fluids was possible and this pellet possessed an enzyme activity capable of utilizing poly(rCm).oligo(dG).

Sucrose density gradient centrifugation of these pellets resulted in the observation of two peaks of polymerase activity at 1.14 g/ml and 1.20 g/ml buoyant densities. ³²P-labelled material shows two peaks of coincident density.

³²P-labelled RNA was prepared from this labelled material of both 1.14 and 1.20 g/ml density and similar experiments carried out as those for uninduced cells. The ³²P-RNA show identical characteristics to the uninduced Drosophila cell 1.22 g/ml ³H-RNA i.e. a 60-70S complex may be isolated and, if the RNA sample is heat denatured before analysis, a 30-35S moiety is isolated with the disappearance of the 60-70S species. Again the relationship of the 30-35S species

to the 60-70S species is unclear.

If yields are increased both proteins and nucleic acids may be analysed and compared to the known Retroviridae. Sequence homology with other RNAs may be established for example with other retroviruses and with the genetic element Copia (Potter et al, 1979; Strobel et al, 1979; Young, 1979).

Known retroviruses have proviral DNA with several structural features in common with insertion elements eg. the terminal repeat structure described in the Introduction. There is currently much interest in investigating the relationship between these proviral DNAs and insertion elements.

Since the structures of proviruses and transposons are very similar either a) transposons and proviruses have a common ancestor,

or b) they are members of the same "group of sequences".

It is obviously of great importance to determine what the relationship between these two types of genetic element is.

Until now this was extremely difficult to determine since there is no reported system where both proviruses and transposons were present. However Drosophila has an abundance of transposable elements. The best studied of these transposable or insertion, elements is designated Copia.

Copia is a transposable genetic element and is reported to be the most abundant poly(A)-RNA in cultured Drosophila cells (Finnegan et al, 1978). Copia elements are about 5 Kb long and possess terminal direct repeats of 0.3 Kb (Potter et al, 1979). This genetic element therefore shares several characteristics with the genome of the Retroviridae.

Further characterization of the nucleic acid species detected in this work could prove them to be endogenous proviral sequences. This would enable us to study the relationship between the transposons of Drosophila and putative proviral sequences for the first time. A series of hybridizations should clarify the nature of the RNAs isolated from the induced and uninduced Drosophila cells.

These experiments could also help us to understand the relationship between insertion elements and retroviruses.

When avian and murine retroviruses were added to insect cells no satisfactory result was obtained. This was due to the nature of the experiment, assaying for reverse transcriptase post-infection. A better approach is that of using a molecular probe within a well-defined system.

However Southern blot analysis of XLV(KMSV) infection of CAT and Drosophila cells gave surprising results. The XLV(KMSV) virus used in the experiments was isolated from rat producer cells originally infected with XLV(KMSV). It is known that C-types produced from rat cells can pick up other RNAs and DNAs (see Introduction). The KMSV genome contained within the XLV coat may thus change its genetic constitution by picking up extraneous nucleic acids. This may give rise to altered restriction patterns when hybridized with KMSV probe. However it is probable that the XLV/XLV component of the virus stock gave rise to the results with CAT cells.

These "impurities" may be reduced by taking original virus stock and building up titres by using cloned CAT cells, avoiding the question of the origin of acquired sequences.

Results with Drosophila cells demonstrated that these cells could pick up sequences post-infection with a retrovirus. The exact origin of these sequences could not be determined and further experiments are required.

Infection of cells with the induced culture fluids from Drosophila cells was also followed by rAdT utilization in extracellular culture fluids. Negative results obtained are open to the same criticisms presented for the infection of insect cells by known retroviruses.

A better experimental approach would be to obtain a quantity of RNA from the induced 'Drosophila particle' and synthesize a DNA probe. Unintegrated and integrated 'proviral' DNA could then be assayed. Experiments to determine the extent of infection could then be carried out, eg the Drosophila particle may enter other invertebrate cells and become integrated into the host cell genome but not transcribed.

CONCLUSIONS

Our Drosophila cell line appears to contain intracellular retrovirus-like entities similar, but not identical, to those observed in murine and avian cells.

Extracellular levels of rAdT utilizing polymerase activity can be greatly increased by iododeoxyuridine treatment of cells. This polymerase activity can be resolved into two fractions; one of 1.14 g/ml, another of 1.20 g/ml. The relationship of these fractions to the intracellular particle present in uninduced Drosophila cells is unknown. However RNA isolated from both 1.14 and 1.20 g/ml fractions exhibits characteristics similar to those of the Retroviridae.

Further experiments could be aimed at establishing a molecular probe to the induced Drosophila particle. Amongst various DNAs to be probed wild type Drosophila DNA should be assayed to ascertain the distribution of the particle sequences found as an RNA in the cell line. This would also help to identify the origin of such sequences. The relationship of these sequences to the known transposable elements of Drosophila should also be determined. This could provide valuable information on the origin of Retrovirus sequences. Experiments aimed to determine these relationships are under way.

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